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PREFACE

About the time we think our task is done for the year and we are congratulating ourselves and our authors, we are reminded that it is customary to prepare a Preface to this *Review*. When so much work has been reviewed so succinctly and so well, it truly behooves us to bestir ourselves and express publicly our gratitude to those who have collaborated in the authorship of this volume. Their surveys of advances in various fields of microbiology lead to a better understanding of life in both its micro- and macro- forms.

We regret that factors beyond our control have made it impossible to include in this issue the scheduled review on heterocaryosis. The chapter on chlamydomonas, genetics and cytology, which was planned for Volume 13, is to be deferred until 1960 as is also the review on fine structure of virus-infected cells.

At this time we wish to express our thanks to Dr. Tracy M. Sonneborn for his efforts on behalf of this Review. We are pleased to announce the appointment of Dr. Theodore L. Jahn of the University of California, as Dr. Sonneborn's successor on the Editorial Committee.

Once again, we express our deepest appreciation for the valuable assistance and cooperation of the office staff of Annual Reviews, Inc., acknowledging, in particular, the most capable efforts of Miss Beryl V. Daniel as editorial assistant (and ring master).

S.P.C. T.L.J. C.E.C. S.R. C.A.E. M.P.S. R.E.H. W.B.U.



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BIOSYNTHETIC ASPECTS OF METABOLISM^{1,2}

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INTRODUCTION

A complete review of synthetic aspects of metabolism would include almost every biochemical event, as few can be found which are not related in some way to the flow of material and energy needed for biosynthesis. For obvious reasons, such a comprehensive review is not possible; we have therefore chosen a much more limited range, the biosynthesis of macromolecules wherein the transfer of information is an important and still obscure feature. These include deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. During the year there has been a considerable advance in understanding the composition and properties of cell wall, cytoplasmic membrane, and ribonucleoprotein particles (ribosomes). As it is often necessary to distinguish what type of protein or RNA is being synthesized we considered it desirable to include some new data on the composition of these structural elements. We have also included certain experiments which indicate how synthesis of both large and small molecules is selectively controlled to maintain a balanced growth of the cell. Energy-yielding reactions and the synthesis of small molecules have been excluded except where they yield information on the subsequent synthesis of larger molecules.

In arranging the wide variety of reports falling within these limits we have attempted to cover first those synthetic reactions which can be carried out with purified enzyme preparations; second, the reactions which are observed with cellular fragments; third, reactions of intact cells and, finally, the changes in synthetic reactions which are caused by various disturbances. As might be expected, the papers do not fall neatly into one category or another. In listing the references we have omitted preliminary reports if a complete report covering the same material was available.

 $^{\rm 1}$ The survey of the literature pertaining to this review was concluded in December, 1958.

² The following abbreviations will be used: ADP (adenosine diphosphate); AMP (adenosine monophosphate); ATP (adenosine triphosphate); DNA, DNase (deoxyribonucleic acid, deoxyribonucleic acid, deoxyribonucleic acid, deoxyribonucleic acid); EDTA (ethylenediaminetetraacetic acid); GDP (guanosine diphosphate); Poly A (polyadenylic acid); Poly C (polycytidylic acid); Poly I (polyinosinic acid); Poly U (polyuridylic acid); Poly AGUC (mixed polymer); RNA, RNase (ribonucleic acid, ribonuclease); RNP (ribonucleoprotein); TCA (trichloroacetic acid); UDP (uridine diphosphate).

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BIOSYNTHESIS BY PURIFIED ENZYMES

DNA SYNTHESIS

Full details of the DNA-polymerizing system reported earlier by Kornberg (1, 2) have been published. Preparation of the deoxynucleoside triphosphates, required as substrates, was carried out using deoxynucleotides isolated from digests of P32-labeled DNA derived from Escherichia coli. These were separated by chromatography on Dowex-1 and phosphorylated by ATP to the corresponding triphosphates, using a deoxynucleotide kinase partially purified from extracts of E. coli. The triphosphates were then purified by chromatography on Dowex-1. The polymerase was purified from extracts of E. coli by a procedure which included streptomycin precipitation, DNase digestion, alumina gel adsorption, ammonium sulfate fractionation, and chromatography on diethylaminoethyl cellulose (DEAE). Bacteria other than E. coli gave lower activities and acetone powder extracts of calf thymus or HeLa cells were one hundred times less active (3). The enzyme required the presence of all four deoxynucleoside triphosphates, together with a DNA primer for synthesis of DNA. Tenfold or greater increases in the DNA content were observed. Pyrophosphate was released by the polymerizing reaction (4). The product was DNA of molecular weight 5×10⁶ as shown by chemical and physical studies (5, 6). The reverse reaction required high concentrations of pyrophosphate and was only slightly inhibited by omission of a single nucleotide (4). The product, DNA, had a base ratio depending on and resembling that of the primer (7). Incomplete purification of the enzyme left a contaminating enzyme which split deoxyguanosine triphosphate to the nucleoside (8). The incorporation of single deoxynucleoside triphosphates into DNA was shown to result from attachment at the deoxynucleoside end of the DNA chain (9). Analogues were incorporated specifically; thus, deoxynucleoside triphosphates of uracil and 5-bromouracil were incorporated in place of thymine; 5-methyl and 5-bromocytosine in place of cytosine, and hypoxanthine in place of guanine (10).

The purified enzyme had a high specific activity and could account for the rate of DNA synthesis in growing cells if one per cent of the cellular proteins were present as this enzyme. Thus, cell-free enzymic systems are capable of carrying out some of the most complicated reactions at rates comparable to those of the growing cell when the appropriate conditions are found. A final proof that this system is, in fact, the one operating to duplicate DNA in living systems, requires only the production of biologically active DNA.

RNA SYNTHESIS

Synthesis of polyribonucleotides by the enzyme polynucleotide phosphorylase has been reviewed (11, 12) and Ochoa and his colleagues have published three papers in a new series dealing with the structure of synthetic polymers containing one or a mixture of bases and of the products of interaction of homopolymers (13, 14, 15). The enzyme from Azotobacter vinelandii

can synthesize polyribothymidylic acid from the ribose analogue of thymidine diphosphate (16). Beers has continued his study of the enzyme from Micrococcus lysodeikticus and reported on the role of magnesium and salt (17). An enzyme system having properties both similar to and different from those of bacterial polynucleotide phosphorylases has been extracted from yeast [(18); see also (19)]. Mii & Ochoa found that a primer was necessary for the functioning of the Azotobacter enzyme (20). A crude extract of A. vinelandii was purified 250 to 300-fold to give a preparation 70 to 80 per cent pure but still containing up to 3 per cent nucleic acid or nucleotide. The crude preparations with 40 per cent nucleic acid formed polynucleotides immediately but the purer preparations showed a lag which could be reduced by adding appropriate "primers." Poly A, Poly U, Poly C, and Poly AGUC syntheses were studied. Various substances primed, inhibited, or had no effect as shown in Table 1. The significance of the results is not known.

 ${\bf TABLE~I}$ Effects of Polynucleotides on Polymerization of Various Substrates

Substrate	Polynucleotide tested for priming action				
	Poly A	Poly U	Poly C	RNA or Poly AGUC	
ADP	+	_	+	+	
UDP	_	+	+	+	
CDP	_	_	+	***	
ADP+GDP+UDP+CDP	0	0	+	+	

+, 0 and - signify primer, no effect and inhibitor, respectively (20).

Smellie [see (11)] found that the relative concentrations of the four nucleoside diphosphates influenced the composition of the product. With equimolar amounts, the resulting Poly AGUC resembled to some extent the RNA of A. vinelandii in base content. Singer studied the breakdown of oligonucleotides by polynucleotide phosphorylase (21). Tri- and tetranucleotides with C'5 terminal phosphate were attacked but those with C'3 phosphate were not.

There have been several studies of complex formation between homopolymers. Warner (15) followed the interaction of Poly A and Poly U to give Poly (A+U). Rich et al. investigated Poly (A+U), Poly (I+C), and Poly (A+I) (22, 23, 24). In addition, it was found that Poly (A+U) could react with more Poly U (but not with Poly A or Poly C) to give a complex Poly (A+2U) and, similarly, Poly (A+2I) could be formed. It is believed that complexes such as Poly (A+U) have a two-stranded helical structure with H-bonding between pairs of bases and a geometry similar to that of DNA. Concentrations of Mg⁺⁺ or Mn⁺⁺ about equal to those of the bases were nec-

essary for their formation. The complex Poly (A+2U) is considered to be three-stranded and the concentration of cation must be 50 to $100\times$ greater for its formation (22). A three-stranded Poly I has also been reported (25). It was suggested that Poly (A+2U) might resemble a single strand of RNA wound around a two-stranded DNA, and Morgan & Bear have discussed the consequences of RNA structure (if it is like that of Poly A) in connection with its ability to bond with amino acids (26).

Biological activity of synthetic polynucleotides has been tested using stimulation of synthesis of streptolysin S by hemolytic streptococci as an index (27). This synthesis was stimulated by yeast RNA but Poly AGUC was even more active. Activity of each was increased by digestion with RNase. Preparations of natural RNA and enzymically-synthesized polynucleotides containing little or no guanine were inactive. From yeast RNA digested with RNase, a fraction was obtained which was five times as active as the starting material and whose guanine content was about double although the adenine, uracil, and cytosine levels were unchanged (28, 29). This material was thought to contain five or six nucleotides and removal of a terminal pyrimidine did not alter its activity. If this is so, the system is perhaps not a very satisfactory one with which to assay the biological activity of synthetic "nucleic acid."

AMINO ACID ACTIVATION

Two recent reviews (30, 31) give many examples of the presence of amino acid-activating enzymes in microorganisms. Further data were given by Novelli (32) at a symposium on amino acid activation held by the National Academy of Science in the United States. It was pointed out that the ATPpyrophosphate exchange and hydroxamate formation are probably not alternative methods of measuring the same reaction. Hydroxamate formation was stimulated by a group of amino acids which caused no increase in the rate of ATP-pyrophosphate exchange. In another paper Cormier & Novelli (33) reported that formation of glycine hydroxamate resulted in the production of ADP and PO4[™]. The enzyme preparation used was extracted from Photobacterium fischerii and it also catalyzed a PO4 -ATP exchange independent of glycine or other amino acids. An ATP-PO₄[™] exchange reaction is also catalyzed by extracts of E. coli. This exchange was accelerated by amino acids some of which were concurrently decarboxylated (34). A tyrosineactivating enzyme was isolated from yeast and a purity of 70 per cent was achieved. The enzyme catalyzed both pyrophosphate exchange with ATP and hydroxamate formation. It was highly specific for tyrosine; even phenylalanine was inactive (35).

Aminoacyl adenylates seem to be intermediates in the pyrophosphate-ATP exchange reactions and the formation of labeled valyl-AMP was demonstrated by using C¹²-valyl-AMP as a trapping agent (36). However, Berg (37) found that an enzyme specific for methionine in the forward reaction could form ATP from pyrophosphate and a number of amino acyladenylates.

TABLE II
Absolute Rates of Amino Acid Incorporation Calculated From
Published Data

Organism	Fraction	Amino Acid	Rate µM/hr./gm. Protein	Refer- ence
Tetrahymena	Particles and soluble	leucine	.4	(53)
Staphylococcus aureus	intact cells	glutamic acid	8*	(56)
	fragments	glutamic acid	8*	
Alcaligenes faecalis	fragments	leucine	1.5	(57)
		glycine	3.0	
		proline	30	
Bacillus megaterium	intact cells	glycine	15	(58)
	protoplasts	glycine	25	
	lysed protoplasts	glycine	2.1	
	fragments	glycine	3	
Escherichia coli	fragments	leucine	30	(60)
	particles	leucine	< .3	
	soluble	leucine	<.3	
Bacillus subtilis	lysates	glycine	1	(65)
Tetrahymena	kinetosomes		40	(68)
Yeast	particles and soluble	glutamic acid	1	(69)
Rat liver	particles and soluble	leucine	1.6	(70)
Escherichia coli	growing cells (generation time 1 hr.)	glycine	500	

^{*} Incorporation into protein other than cell wall.

A similar lack of specificity in the backward reactions was reported by Novelli (32) for a purified tryptophan-activating enzyme.

Experiments with O¹⁸ have given further information on the mechanism of activation. Bernlohr & Webster found that *Azotobacter* extracts catalyzed a transfer of carboxyl-O¹⁸ from tryptophan, alanine, or a mixture of amino

acids to PO₄[≡] of AMP (38). Boyer & Stulberg (39) used O¹8-labeled amino acids to study amino acid activation reactions in purified enzyme systems to show a transfer of O¹8-from tryptophan to AMP. In vivo experiments were also carried out. Leuconostoc mesenteroides was grown on a medium containing carboxyl-O¹8-labeled amino acids. Ninety per cent of the O¹8 lost from the amino acids was recovered in lactic acid. These experiments were interpreted as giving evidence for the occurrence of amino acid activation in growing intact cells. Boeyé, however, found no activated glycine capable of forming hydroxamate in yeast, whether growing or not, nor in protoplasts of Micrococcus lysodeikticus (40). Growing E. coli yielded leucine and tyrosine hydroxamic acids but only in very small quantities (41).

A transfer of activated amino acids to RNA was demonstrated by Berg & Ofengand (42) using activating enzymes and RNA from extracts of *E. coli*. RNA from other sources and enzymatically synthesized polynucleotides were less than 5 per cent as effective as the *E. coli* RNA as amino acid acceptors. The same enzyme appeared to be involved both in activation and in transfer since these two properties were not separated during the purification of the enzyme. The RNA seemed to have specific binding sites for the amino acids because the incorporation of one amino acid was independent of the incorporation of others.

The formation of RNA-amino acid linkages in subcellular fragments is often easily observed, particularly when the system does not allow formation of completed proteins. These systems are described in Section B. Other evidence for the significance of these processes in the synthesis of protein comes from the study of intact cells. In A. vinelandii Bernlohr & Webster (43) observed a rapid incorporation of C¹⁴-glycine and C¹⁴-leucine into compounds capable of forming hydroxamates. Other reports give evidence for the occurrence of carboxyl-activated peptides in yeast (44, 45) in Streptococcus faecalis (46, 47) and in Bacillus mesentericus and Proteus vulgaris (48). A peptide linked to cytidylic acid found in Penicillium urticae is mentioned by Bergkvist (49) who found a similar compound in Polyporus squamous.

In addition to the activation of amino acids which may be a prelude to protein synthesis, another reaction of significance for biosynthesis has been studied in detail. This is the activation of SO₄⁼ which is a likely prelude to the formation of certain sulfur compounds. Robbins & Lipmann (50, 51) reported the enzymic formation from ATP and SO₄⁼ of adenosine-5'-phosphosulfate which can be subsequently phosphorylated by ATP to yield 3'-phosphoadenosine-5'-phosphosulfate. These reactions and a further transfer of the sulfate to various acceptors were discussed in a summary by Lipmann (52).

SYNTHESIS BY SUBCELLULAR PREPARATIONS

The protozoan, *Tetrahymena pyriformis* has been used by Mager & Lipmann to study amino acid incorporation (53). Fractions equivalent to mitochondria, microsomes, and supernatant were separated and tested singly

and in combinations. Microsomes plus supernatant was the most active combination in fixing leucine into protein but both unsupplemented mitochondria and microsomes had significant activity. Incorporation by *Tetrahymena* microsomes was stimulated also by liver supernatant and vice versa. The protozoal supernatant fixed leucine in a form stable to cold TCA but soluble in hot TCA, dilute alkali, or after RNase digestion. It could also be displaced by addition of pyrophosphate or, better, by pyrophosphate plus AMP; addition of fluoride (to inhibit pyrophosphatase) reduced it still further. The scheme proposed was as follows:

ATP + amino acid

amino acyl-AMP + pyrophosphate

Amino acyl-AMP + polynucleotide

amino acyl-polynucleotide + AMP

AMP by itself did not reverse binding of leucine by the supernatant perhaps because amino acyl-AMP is not a free intermediate.

Gale & Folkes presented full details of the preparation and properties of a factor which stimulated incorporation of amino acids by disrupted cells of Staphylococcus aureus (54, 55). Ultrasonically disrupted cells retained a part of their capacity to synthesize protein and RNA unless treated with RNase, DNase, or 1 M NaCl. The synthetic capacities could be restored after this treatment by addition of RNA, DNA, or their digests. Purification of the digest yielded an "amino acid incorporation factor" which was, per gram, 1000 times as effective as RNA in restoring the synthetic capacities. RNA from E. coli, S. aureus, and Bacillus megaterium have been used successfully as a source of the incorporation factor, B. megaterium being the best source. The isolation procedure involved digestion of the nucleic acid with RNase followed by NaOH, ethanol extraction, ionophoresis, and paper chromatography in two solvents. The chemical nature of the incorporation factor is not yet determined but a number of known compounds have been tested and found inactive. The incorporation factor was as effective as nucleic acid in restoring incorporation of glycine, phenylalanine, aspartic acid, leucine, arginine, glutamic acid, and tyrosine, but gave only a partial replacement when tested with valine, isoleucine, tyrosine, and proline. It was equally effective whether the incorporation of an amino acid was tested singly or with a complete mixture of others. It had no effect on the synthesis of catalase or \(\beta\)-galactosidase.

The incorporation of adenine by disrupted cells was reduced by 98 per cent when the fragments were treated with nuclease. Adenine incorporation by the nucleic acid-depleted fragments was restored by the addition of nucleic acid. Nucleic acid could be replaced by the incorporation factor. The restoration by the incorporation factor was only partial if amino acids were missing or if chloramphenicol were present. When the DNA of the fragments was reduced to very low levels the incorporation factor was less efficient and its action was augmented by the addition of DNA.

Further work on this system (56) showed that amino acids incorporated singly appeared in part in the cell wall fraction (particularly glutamic acid,

lysine, alanine, and glycine) and in part in a compound having some properties suggestive of an RNA-amino acid complex. Electrophoretic studies showed that an amino acid incorporated singly and associated with a single fast-moving component was subsequently found distributed throughout the proteins after a second incubation with all the other amino acids present. The incorporation factor increased both the incorporation of single amino acids into the cell wall and the fast-moving compound and the incorporation of amino acids into the proteins when the presence of other amino acids permitted the process to go to completion.

Alcaligenes faecalis, after sonic disintegration, has been fractionated into particulate material which contained fragments of the cell membrane and a supernatant fraction (57). Beljanski & Ochoa found that unwashed fragments could incorporate all amino acids tested but that washing with 1.0 M NaCl reduced this capacity markedly. The supernatant fraction restored activity and appeared to contain two components, one thermolabile which was purified 800 times and called the "incorporation enzyme," and a thermostable component which might have been RNA. The enzyme was distinct from amino acid-activating enzymes and from polynucleotide phosphorylase. The incorporation of labeled amino acids into protein was dependent on the presence of O₂, was prevented by dinitrophenol, and was linear for 60 min. Washed cell fragments could be reactivated by adding a complete mixture of amino acids or the activating enzyme but there was little additive effect. The rates of incorporation of different amino acids were widely different with proline and threonine eight and five times as rapid as the next fastest, glycine and lysine. The others were two to four times slower. Addition of all the amino acids and the incorporation enzyme to washed cell fragments appeared to result in a 20 per cent net increase in protein but in the absence of amino acids, the increase was half this amount and without either enzyme or amino acids, only one-third as much. These increases in protein in the absence of obvious precursors have not been explained.

Experiments with intact cells, intact and broken protoplasts, and subcellular fractions of *B. megaterium* gave indications that the cytoplasmic membrane was active in protein synthesis (58). Incubation of whole cells with various C¹⁴-amino acids showed lower specific radioactivities in wall fractions than in the residual protoplast. Similar experiments using C¹⁴-glycine showed specific radioactivities which were initially higher in the cytoplasmic membrane and subsequently higher in the cytoplasm.

Incorporation of amino acids into protoplasts showed a similar time course. Incorporation into the cytoplasmic membrane (but little incorporation into cytoplasm) occurred in broken protoplasts. The membrane fraction obtained from broken protoplasts also fixed amino acids. Neither the method of breaking the protoplasts, washing, storage for two days, nor treatment with DNase had any appreciable effect on the ability of the membrane fragments to incorporate amino acids. Little incorporation into the separated cytoplasmic proteins could be found unless they were incubated with

labeled membrane fractions. In this case the label previously incorporated in the membrane fraction was transferred to the cytoplasm. The transfer was greater the shorter the period used to incorporate tracer into the membrane (58).

Any interpretation of the kinetics of these experiments in terms of precursor-product relationships is difficult. The fractions obtained contain both precursors and products so that simple relationships would not be expected. Furthermore, the rates of incorporation observed with the whole cells and their protoplasts were far from optimal so that steady-state conditions cannot be assumed. Even the transfer of activity from membrane to cytoplasm is open to the alternative interpretation that enzymes of the cytoplasm solubilized a part of the membrane.

Spiegelman and his colleagues who earlier reported results with osmotically shocked protoplasts of *B. megaterium* (59), have now turned to subcellular fractions separated from lysates of penicillin-induced spheroplasts of *E. coli.* (60). Three fractions were differentiated, a "membrane fraction" containing protein, RNA and DNA; a high-speed pellet containing the bulk of the ribosomes; and a supernatant containing most of the soluble protein. Kinetic experiments with intact spheroplasts indicated that the sequence of labeling by C¹⁴-leucine was membrane fraction first, followed by soluble protein, and then particles. The membrane was also most rapidly labeled from C¹⁴-uridine and a pulse of this tracer passed into and out of this fraction and on into the others. The particle and supernatant fractions had amino acidactivating activity and could incorporate amino acids but by linkages which were sensitive to hot TCA and RNase. Such incorporation was not inhibited by chloramphenicol. The particles could later bind amino acids in a form stable to hot acid.

The membrane fraction, however, had incorporating ability 100 times that of the particles and supernatant and it seemed that amino acids went almost exclusively into hot acid-stable linkages. Optimal conditions required the presence of all the amino acids, all four ribonucleoside triphosphates, all four deoxyribonucleotides, and $Mn^{++}(5\times10^{-3}M)$. This system was sensitive to chloramphenicol (200 μ g./ml. gave 93 per cent inhibition of leucine incorporation).

It was also observed that the membrane fraction could synthesize polyribonucleotide and that concomitant degradation could be prevented by adding spermine $(5\times 10^{-3}M)$. Optimal synthesis was obtained in the presence of all four ribonucleoside triphosphates, four ribonucleotides, four deoxyribonucleotides, Mg^{++} $(1.0\times 10^{-3}M)$, and Mn^{++} $(5\times 10^{-3}M)$. Omission of ATP or Mn^{++} or of the eight nucleotides (leaving, however, the four triphosphates) abolished activity; omission of the three triphosphates other than ATP or replacement of these by the corresponding riboside diphosphates markedly reduced polynucleotide synthesis. When ATP was the only triphosphate present it seemed that a polyadenylate was formed. Both RNase and DNase inhibited synthesis of mixed polynucleotides but only the for-

mer caused breakdown of the product; the formation of polyadenylate was comparatively insensitive to RNase. The system thus differs in many respects from the polynucleotide phosphorylase which uses diphosphates and is activated by Mg⁺⁺ (11, 12). The membrane fraction is capable of increasing its protein content by 15 per cent and its polynucleotide by 1000 per cent in 3 hr. It is also reported to be able to synthesize an inducible enzyme. Such properties make it an extremely useful experimental system.

Perhaps related to these results is the finding that lysates of $E.\ coli$ spheroplasts formed by lysozyme/EDTA treatment were able to make tryptophanase (61). After a lag, DNA was also formed. The thymine-less strain 15 T⁻ required thymine both for DNA synthesis and for enzyme formation. 6-Azauracil and 5-bromouracil (10 to $20\ \mu g./ml.$) prevented enzyme formation but much higher concentrations of 6-azathymine had little effect. It would be interesting to know the exact procedure used in these experiments since it is well established that EDTA can disrupt ribosomes and that Mg⁺⁺ and Mn⁺⁺ are essential in many comparable systems. [See also (62, 63)].

The ability of $E.\ coli$ to make β -galactosidase has been studied after growth in penicillin which induces spheroplast formation (64). Samples for assay of enzyme-forming capacities were taken after various periods of growth, washed and resuspended in cold 1 per cent NaCl in a tight-fitting glass homogenizer before being incubated in the induction medium. During growth in penicillin the cells became nonviable and osmotically fragile; the enzyme-forming ability decreased to a minimum of about 10 per cent (at 30 min.) but then rose again to its intial value (at 50 min.) before declining once more. Spheroplast formation occurred during the period when the activity was rapidly rising and was accompanied by massive synthesis of DNA (an eightfold increase between the 20th and the 60th min.). Such rates of DNA synthesis have previously been observed only in phage-infected systems.

Polyvinyl sulfate and a protease inhibitor prepared from potato extract were found to maintain the amylase-forming capacity of lysates of *B. subtilis* (65). Rates of enzyme formation and of glycine incorporation into protein and nucleic acid approximately equal to those of unlysed cells were observed. The rate of glycine incorporation into the control culture was, however, only 1/500 of that which might be expected in a growing culture. The effect of the polyvinyl sulfate was attributed to its inhibition of RNase activity (65). The amylase formation was inhibited by 8-azaguanine but not by dinitrophenol (66).

Mechanically damaged preparations of *Pasteurella pestis* (sonic treatment but not Hughes' press or Mickle disintegrator) were reported to be able to form antigenic material. The debris and a component of the supernatant were both necessary. The latter was nondialyzable and was resistant to trypsin, RNase, and DNase (67).

Another system, which preliminary reports indicate may have considerable synthetic ability, consists of the isolated kinetosomes of Tetrahymena

pyriformis. These organelles which lie at the base of the cilia have been separated and found to synthesize protein at about 8 per cent of their own dry weight per hour when supplemented with amino acids, purines and pyrimidines, growth factors, and a source of energy (68).

Webster compared the incorporation of C^{14} -glutamic acid by preparations of ribosomes from yeast and from pea seedlings (69). The system employed was very similar to those used to study the *in vitro* incorporation of amino acids by the microsome fraction of mammalian tissues. The presence of particles, "pH 5 enzymes," ATP, ATP-generating system, guanosine triphosphate, and magnesium were required for full activity. A lower concentration of ATP ($10^{-4}M$) and magnesium ($10^{-5}M$) than used with the liver microsomes was needed as higher concentrations were inhibitory. The time course of incorporation was stated to be similar to that observed with liver microsomes. The rate of incorporation was small, $\frac{1}{3} \, \mu \text{mol/gm}$, protein/hr. In view of Spiegelman's observation that incorporation is extremely small in the ribosome fraction as compared to the fraction containing membrane fragments (70), it seems possible that the observed effect could arise in part from contamination by membrane fragments.

BIOSYNTHESIS IN WHOLE CELLS

METABOLIC POOLS

The nature and state of the fraction readily extractable from cells continues to be studied. Accumulation of galactosides by spheroplasts of E. coli suggested to Sistrom that the major fraction of this pool was osmotically active (71). Hancock investigated quantitatively the release of free amino acids from Staphylococcus aureus by various treatments. Results of several extraction procedures indicated a level of 400 to 500 µmoles amino acids per gram dry weight and this was not altered by washes ranging from distilled water to 1.0 M NaCl or 1.0 M sucrose. All of the protein amino acids were present but their relative amounts were unrelated to their relative abundances in the protein. Glutamic acid, aspartic acid, and proline levels were highest and, in exponentially growing cells, all amino acids were more concentrated (1.5 to 25 times) than in the external medium. No amino sugars were detected but an unknown dialyzable component of the pool was found on hydrolysis to give only those amino acids which occur in the cell wall of S. aureus, i.e., glutamic acid, alanine, lysine, and glycine. No amino sugar, ultraviolet-absorbing material, or phosphate was released, however (72).

Gerhardt et al. carried out balance studies on lysine uptake by intact cells and isolated protoplasts of *Micrococcus lysodeikticus* which showed that although the pool which could be released from protoplasts was at least as large as that from cells, the cell wall could bind large amounts of lysine (73, 74). This was only partly removed by washing with water but the binding was reversible at pH values near the isoelectric pH of lysine.

Mandelstam studied the pools in growing and nongrowing cultures of

E. coli and obtained results consistent with the hypothesis that the free amino acids of the pool are precursors of protein (75). Hancock, likewise, concluded that in S. aureus exogenous proline passed through the pool before being incorporated into protein (76). Kinetic studies of amino acid uptake into the pool and protein of yeast led Halvorson & Cohen (77) to suggest that exogenous amino acids could be used preferentially for protein synthesis without equilibrating with the pool. Cowie & McClure, however, obtained evidence that the amino acid pool of Candida utilis consists of an exchangeable, "expandable" part and an "internal pool" which is an obligatory intermediate in protein synthesis (78). These conflicting conclusions may perhaps be resolved if a model is considered in which exogenous amino acid after penetrating the cell membrane can pass either into the "expandable" pool or into a form more directly in the pathway of protein synthesis. Nevertheless, it is still unclear how relatively large amounts of amino acids can be held within the cells in a form from which they can be instantly released by a variety of physical and chemical procedures and yet not be exchangeable with exogenous amino acids even under conditions when these are being rapidly taken up into the "expandable" pool. The alternative theories of pools held by permeability barriers on the one hand, and by adsorption on the other, both have favorable and unfavorable implications.

The problems of the nature of the amino acid pools in *E. coli* are related to but not identical with those of yeast. A discussion of the consequences of the permease theory, stoichiometric adsorption site theory, and theories involving a carrier and an adsorption site has appeared (79). No single model was found to be entirely satisfactory in interpreting a wide range of experimental observations concerning the pool of *E. coli* but the carrier plus site

mechanism appeared to be the most adequate.

As binding of amino acids to macromolecules is postulated in many models of the pool, the following three studies are pertinent. Cornwell & Luck studied the binding of amino acids to insulin and histones (80); Zubay & Doty observed binding of amino acids to nucleic acid (81) as did Mark & Stauff (82).

The nature of other components of pools has been investigated less frequently. Cowie & Bolton used C¹⁴-adenine and C¹⁴-guanine to study pools of nucleic acid precursors in growing *Candida utilis*. Both a purine pool, the size of which depended on the external purine concentration, and a purine nucleotide pool which was independent of the external concentration were found. Conversion of adenine to guanine as well as incorporation into nucleic acid occurred after conversion to nucleotides (83). In addition to the usual nucleotide pool of yeast, a peptide derivative of uridine-5'-pyrophosphate has been reported. Hydrolysis yielded aspartic acid, glutamic acid, arginine, and alanine (84).

Extraction of yeast first by alcohol and subsequently by TCA was found to yield two fractions of quite distinct characteristics. The alcohol-soluble fraction contained nucleotides which decreased in quantity as the cells started to grow. In addition, activated peptides were present; these increased as growth started. The TCA-soluble pool also increased at the onset of growth. It contained a multiplicity of compounds not yet identified (45).

Mudd et al. observed accumulation of polyphosphate in Mycobacterium chelonei and M. thamnopheos. The polyphosphate served as a phosphorus reserve which could later be used in RNA synthesis (85).

PEPTIDES

Further reports have appeared on antagonistic effects of peptides and on inhibitions caused by amino acids and their analogues. Growth of Lactobacillus arabinosus and induced synthesis of malic enzyme were studied by Shive et al. who investigated inhibition of the utilization of keto-valine, valine and a valine peptide by keto-isoleucine, isoleucine and an isoleucine peptide (86). They concluded that three separate pathways exist for the formation of "active" valine. Similar deductions were made in the case of phenylalanine, its keto analogue, and a peptide, in experiments involving β-2-thienyl-DL-alanine and the glycyl peptide of this (87, 88). Inhibition of growth of lactobacilli by a lysine analogue, S-(β-aminoethyl) cysteine, was overcome by lysine peptides (89). The streptogenin-like action on growth of Lactobacillus casei shown by the pentapeptide Ser-His-Leu-Val-Glu was competitively antagonized by some other peptides including one in which threonine replaced the serine of the pentapeptide (90). Growth of a mutant of Bacillus subtilis was found to be stimulated by glycine peptides, apparently as a result of the reversal of an inhibition induced by histidine (91).

There were also reports of the occurrence of peptides in microorganisms. Connell & Watson separated 43 peptides from alcoholic extracts of *Pseudomonas hydrophila*. Kinetic labeling experiments showed that the specific radioactivity of amino acids, peptides, and proteins increased in this sequence (92). While it is unlikely that the peptides were derived from protein, the converse is not necessarily indicated by these data. The measured pool size should result in a considerably more rapid increase in specific radioactivity of the peptides if these were protein precursors. The authors point out that about one-third of the peptides contained diaminopimelic acid and it may be that some are concerned in cell wall synthesis. A leucine peptide which had the same specific radioactivity as free leucine in the pool was reported in alcoholic extracts of *Torula utilis* grown for a short time on C¹⁴-acetate (93).

Biosynthesis of the antibiotic peptide, gramicidin-S, during certain phases of growth apparently involved use of endogenous sources of amino acids and only smaller amounts of exogenous material (94). Pollock & Kramer failed to find evidence of intermediates between free amino acids and bacterial penicillinase and concluded that the lag in enzyme formation was not caused by formation of precursors of which there could not have been more than 400 molecules per cell (95).

The finding of activated peptides (44 to 49) is also relevant to considera-

tions of intermediates in protein synthesis. It is noteworthy, however, that bacterial cell walls contain mucopeptide components, the synthesis of which, while it involves the coupling of amino acids, appears to differ in important respects from the synthesis of protein. None of the kinetic studies of peptide and protein labeling so far reported gives convincing evidence of a precursor-product relationship.

GROWTH OF CELLS

Microorganisms in a suitable environment should continue to grow indefinitely but different kinds of organisms require different conditions and different conditions can result in changes in the growth rate of a particular species (96). Hancock found that the optimum conditions for growth of a thermophilic bacillus were similar to those for optimal incorporation of glycine into protein and for synthesis of catalase and the maltozymase system. It was noted that loss of enzymic activity could occur without degradation of protein (97). Two excellent reports have appeared on the growth kinetics of Salmonella typhimurium (98, 99). Cultures were grown under approximately steady-state conditions in various media and at different temperatures by diluting cultures with equal volumes of fresh medium at intervals corresponding to the mean generation time. The dependence of cell size and composition on the temperature and the nature of the medium was studied first (98). Both were found to be independent of temperature but were determined by the composition of the medium. In rich media the cells were larger, grew more rapidly, and contained more ribonucleoprotein per nuclear unit. Calculations involving reasonable assumptions suggested that the rate of protein synthesis was a direct function of the number of ribonucleoprotein particles at all growth rates. Transfer of steady-state cultures from poor to rich media (and vice versa) demonstrated that adjustment to a new cellular composition occurred in a well-ordered sequence (99). In a shift from low to high growth rate, RNA synthesis rapidly increased, followed about 20 min. later by increase in mass and later still (70 min.) by an abrupt increase in the rate of DNA synthesis and cell division. The effect of these changes was to alter the size, RNP content and DNA content of each cell to that characteristic of the new medium and rate of growth. Conversely, a shift from high to low rate of growth resulted in continued cell division and DNA synthesis without concomitant increase in RNA and mass until the new characteristic composition and growth rate had evolved. These experiments help in the interpretation of older observations on cell size during different phases of growth and in different media and on adaptation to media of altered composition.

When penicillin was added to cultures of Alcaligenes faecalis growing exponentially in tryptone medium the cells became converted to a globular form which had a lowered growth rate (100). Decrease in the rates of RNA, DNA, and protein synthesis occurred at about the same time and the new exponential rate was rapidly established. The data do not justify the con-

clusion that change in DNA rate occurred later than that of RNA and protein.

The interrelations of the synthesis of RNA, DNA, and protein have also been studied in systems artificially synchronized with respect to the time of cell division. Temperature shifts have often been used to induce synchrony and in the case of E. coli it was found that cooling to 4°C, for 30 to 60 min. followed by rewarming to 37°C. was effective. The same result was achieved if cultures were incubated for 4 hr. after the glucose of the medium was exhausted. This caused a decrease in the RNA/DNA ratio to about onethird of that in growing cells and a relatively long lag before synchronous division occurred in fresh media (101). Campbell and Maalge have reviewed the procedures for producing, and the consequences of, synchronous growth of microorganisms (102, 103). The latter author, in particular, points out that most methods of induction of synchrony inevitably involve disturbances in the normal patterns of synthesis of cellular components. Perhaps mechanical methods of segregating cells such as filtration procedures (104, 105) may be least unsatisfactory. No coherent picture of the time course of RNA, DNA, and protein synthesis has yet emerged from these studies.

The growth of single cells of Saccharomyces cerevisiae was observed by interference microscopy (106). The results suggested that individual cells grow at a rate which is linear with time and that exponential growth is a property of cultures rather than cells. This seems to imply that the catalytic apparatus synthesized by a cell is not optimally functional until the cell divides.

DNA DUPLICATION

The Watson-Crick model of DNA structure has suggested a mechanism for the biosynthesis of exact duplicates of molecules. Several possible variations of this mechanism are discussed in a recent review (107). Important experimental evidence for the operation of such a mechanism in growing *E. coli* has been published this year. Meselson & Stahl (108) used a CsCl density gradient in the analytical centrifuge to observe the distribution of N¹⁵ among molecules of DNA following the transfer of uniformly N¹⁵-labeled *E. coli* to a growth medium containing N¹⁴. Until one generation time elapsed, DNA fully labeled with N¹⁵ and N¹⁴ in equal quantities accumulated. Subsequently, only half-labeled DNA and unlabeled DNA were found. From these results the authors conclude (a) The nitrogen of a DNA molecule is divided equally between two subunits which remain intact through many generations; (b) following replication each daughter molecule has received one parental subunit; (c) the replicative act results in a molecular doubling.

The width of the DNA band indicated that the DNA units were monodisperse with a molecular weight of 7.1×10^6 . On heating (100°C., 30 min.) the DNA of *E. coli* dissociated into two molecules of one-half the initial molecular weight. When the half-labeled DNA was thus treated one of the

resulting halves was found to be fully labeled with N¹⁵, whereas the other contained N¹⁴ only. The results of these experiments are in exact accord with the expectations of the Watson-Crick model for DNA duplication.

Painter, Forro & Hughes (109) used H3-labeled thymidine to follow the transfer of DNA from mother to daughter cells. The thymidine-requiring mutant was first grown with tritiated thymidine and then transferred to an unlabeled medium. The distribution of tritium in the progeny was observed by radioautography of single cells. These were taken at random from a culture or from cells isolated by micromanipulation and allowed to pass through a number of divisions. The observed distribution of label among the daughter cells was heterogenous. Divisions wherein one daughter cell received all the label and the other none were observed in the third generation and later, but not in the first division. Other types of division ranging from the extreme of all-or-nothing to random were also observed. This preliminary report does not provide quantitative data on the distribution of the various types of division and the authors do not attempt to interpret their results in terms of a model. It is obvious, however, that a single bacterium contains 500 to 1000 DNA units of the 7×106 mol. wt. size observed by Meselson. Were these to segregate independently, no all-or-nothing divisions would be observed; a much larger organization of DNA must therefore be preserved through division.

Radiation effects (see below) also point to a large unit of DNA, the integrity of which is essential. The localization of DNA within the cell is therefore of great interest. Caro, van Tubergen, & Forro used radioautography of sectioned bacteria labeled with tritiated thymidine and electron microscopy to show that the DNA was confined to the central region of E. coli (110). In contrast, Plaut & Sagan found tritium from tritiated thymidine (which could be removed by DNase but not by RNase) uniformly distributed throughout the cytoplasm of Amoeba proteus (111). Catlin & Cunningham found extracellular DNA, having the same base ratios as the intracellular DNA, accumulating as a slime layer under certain cultural conditions (112). The transfer of P32-labeled material (possibly (DNA) from one cell to another during mating of E. coli was observed by Garen & Skaar. The quantity of P32 transferred was less than that of one nucleus (113).

Spiegelman, Aronson, & Fitz-James described methods for isolating nuclear bodies from protoplasts of *B. megaterium*. The protoplasts, after formation in a sucrose-phosphate medium, were transferred to a succinate-citrate medium thereby causing a condensation of the chromatinic material. Lysis of the protoplasts by lipase followed by differential centrifugation yielded a fraction containing nuclear bodies. The nuclear bodies, after repeated washing in succinate-citrate buffer, contained DNA, RNA, and protein in the ratio 1:1:3. They appeared to consist of a core of nonchromatinic material surrounded by chromatin and were similar to the nuclear bodies observed in the intact protoplast (114).

Studies of the condensation of chromatin in vegetative cells and in protoplasts were described by Fitz-James (115). Vegetative cells, either growing in peptone medium or after washing in 0.1 M phosphate buffer, showed dispersed chromatin. After transfer to sucrose-phosphate or citrate buffer there was a marked contraction of the chromatin. The contraction was transitory in the sucrose-phosphate medium, being maximal 2 to 6 min. after transfer. (A temporary cessation of protein and nucleic acid synthesis after transfer of E. coli to media containing 0.5 M sucrose has also been observed (41). These two effects may have a significant relationship.) Subsequent removal of the cell wall by lysozyme did not affect the degree of chromatin aggregation but did cause a loss of 12 per cent of the RNA (115). It seems likely that a similar contraction of the nuclear bodies would occur during the dehydration which is a prelude to embedding for sectioning. Cell sections [as in (110) above] may show a smaller nuclear region than actually exists in growing cells.

Further studies compared growth of protoplasts to the growth of vegetative cells. The protoplasts did not divide but the number of nuclear bodies increased. DNA and protein increased at roughly the same rate as did the cell volume and lipide P increased with the cell surface. The RNA synthesis, in contrast, proceeded at a lower rate and the RNA concentration

dropped (116).

TURNOVER OF PROTEIN AND NUCLEIC ACID

It has been known for some time that the proteins and nucleic acids of growing microorganisms are quite stable but that they are slowly lost to the medium when the cells stop growing. It had not been established with certainty whether the losses observed were caused by lysis of a small proportion of the population or whether they arose from partial degradation of the whole population. Reports have appeared this year giving further information on this subject.

Mandelstam found little loss of protein from growing E. coli but a turnover of 4 to 5 per cent per hour in nongrowing cells. This turnover could be

inhibited by chloramphenicol or azide (117).

Halvorson measured the transfer of protein bound C¹⁴-glycine to nucleic acid purine in growing yeast and found a rate of 0.028 per cent per hour (118). In yeast lacking exogenous energy and nitrogen sources, he found a degradation rate of 0.66 per cent per hour for protein and 0.15 per cent for nucleic acid. The degradation was inhibited by azide, dinitrophenol, and arsenate. Lysis of individual cells was ruled out by viability measurements and by the appearance of the breakdown products in the intracellular pools before their appearance in the medium (119).

Borek, Ponticorvo & Rittenberg used the incorporation of O¹⁸ and H² from water to indicate the rate of turnover. In a mutant whose growth was blocked by lack of an amino acid they found a turnover rate of 3 to 6 per cent per hour (120). It is gratifying to find that concepts based on much cruder experiments [cf. (121)] are still valid when tested by these more

elegant techniques.

Breakdown and loss of nucleic acid from Lactobacillus arabinosus was

observed when the cells were incubated in buffer or buffer with glucose. The greatest loss occurred from early lag cells incubated without glucose (122). A similar loss from *Rickettsia mooseri* was also reported (123).

RIBOSOMES

A collection of papers on various aspects of ribonucleoprotein particles (ribosomes) has recently been published (124). Work with mammalian systems has shown a role of the ribonucleoprotein particles of the microsome fraction in protein synthesis (125, 126). During the past year there has been a marked increase in studies of the particles of microorganisms. Chao found a reversible dissociation of 80S ribonucleoprotein particles of yeast into 40S and 60S particles depending on the magnesium content of the suspending fluid (127). A similar effect of magnesium on bacterial ribosomes was reported by Gillchriest & Bock (128) using Azotobacter vinelandii, and by Bolton, Hoyer, & Ritter (129) using E. coli. Other reports of the necessity for magnesium to stabilize the ribosomes from mammalian tissues (130) and from pea seedlings (131) indicate a similarity in the structure of particles independent of their source.

Further information on the role of magnesium was given by Tissières & Watson who found a reversible dissociation of 100S particle from *E. coli* into 2 particles of 70S. The 70S particles in turn dissociated into 51S and 32S components (132).

Purified preparations of particles derived from microorganisms contain RNA and protein. The proportions reported range from 25 per cent to 60 per cent RNA (132 to 135). The enzymic activities previously reported in pellets sedimented by high centrifugal forces appear to be caused by contaminating protein (132, 134, 135, 136).

Dagley & Sykes reported marked changes in the particles of *E. coli*, depending on the state of the cells. In their experiments the 40S peak was prominent in extracts of growing cells but it disappeared on incubation of the cells in phosphate buffer. As their system lacked sufficient magnesium the larger peaks were not observed (136).

Ashikawa observed a predominance of the 80S peak in nitrogen-starved yeast with smaller particles appearing after growth was initiated (137). The 100S component was predominant in *E. coli* when growth was stopped by lack of nitrogen or carbon and a more complex pattern appeared during rapid growth. Characteristic changes in the pattern are also produced by chloramphenicol (79). These changes are apparently correlated with different rates of protein synthesis: the contents of various types of ribosomes may therefore provide a better clue to the role of RNA in protein synthesis than does the total cellular content of RNA.

Ribonuclease was observed in purified ribonucleoprotein by Elson who found that the enzyme was not active unless the integrity of the particles was destroyed by urea, thus implicating the presence of hydrogen bonding (138, 139). Entirely similar results were obtained by Bolton who also showed

that the ribonuclease activity followed the particles in ion exchange chromatography (diethylaminoethyl cellulose) before but not after treatment with urea or EDTA (79, 140). More recent work showed that the particles also contain an enzyme with amino peptidase activity but this enzyme was active without disruption of the particle (141). 70S Particles were dissociated and the resulting 51S and 32S particles were separated by centrifugation. All three types of particles had the same ratio RNase: peptidase: protein: RNA. It is provocative to find two catabolic enzymes located in a particle which is commonly believed to be active in synthesis. A reverse action of these enzymes while held in the structure of the particle seems possible.

Incorporation of P³² into the particulate and nonparticulate RNA of E. coli was measured by Wade & Lovett (142). Cells were exposed to the tracer for 1/5 generation time and then broken and separated into fractions by centrifugation. These fractions were then subjected to chemical fractionation and the specific radioactivities were measured. A number of components were obtained of different specific radioactivities but the incorporation data

are not sufficient to reveal any precursor-product relationships.

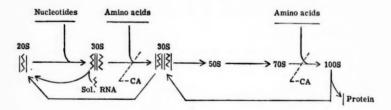
Roberts, Britten & Bolton (79, 135) observed the incorporation of C¹⁴-amino acids, S³⁵O₄[∞] and P³²O₄[∞] into the particles of *E. coli*. Ion exchange chromatography on modified cellulose (diethylaminoethyl cellulose) was used to separate the ribonucleoprotein components. This procedure provided a good separation of the particulate nucleoprotein from free RNA and from contaminating proteins but it caused degradation of the particles as they contained only one-half as much protein after elution. The elution pattern from the column showed an interaction between the column and the nucleic acid of the particles indicating quite a different structure from that of viruses in which the nucleic acid is effectively covered by protein (79).

After short exposures to radioactive amino acids or to S³⁵O₄^m, the specific radioactivity of the nucleoprotein eluted from the column was low compared to that of soluble proteins showing that these structural proteins could not be precursors of the soluble protein. Further experiments have shown that the specific radioactivity of the protein which was still associated with nucleic acid after elution from the column was less than one-half that of the protein of intact particles. Thus, the easily removed portion of the protein appears to be built from free amino acids and accreted to a structure containing pre-existing protein. It should be pointed out that these experiments measured the synthesis of the particles, whereas the early incorporation of tracers into particulate fractions observed *in vivo* with animals is undoubtedly caused by the presence of nonparticle protein synthesized by the particles.

Column chromatography also revealed precursor-product relationships in the synthesis of the nucleic acid portion of the ribosomes. It was reported that P³² appeared first in macromolecules which were eluted at a higher salt concentration than were ribosomes. Thus, the column distinguished the precursor material from the nonparticulate RNA and from the bulk of the

particulate RNA. This precursor could be degraded by RNase and it sedimented in the swinging-bucket centrifuge like a particle of 20–40S. Its behavior on the column indicated that it had less protein than the completed particle. A material of similar properties accumulated in the presence of chloramphenicol which prevented all entry of P³² into completed ribosomes. The soluble RNA was a stable end product of synthesis and not a precursor of the ribosomal RNA.

Recent work (143, 144), using centrifugation to separate the small (20S, 32S, 51S) particles from the larger (70S, 100S) followed by chromatography of the separated fractions, showed that soon after the addition of P³² there were large differences in the specific radioactivities of the different particles. The small ribosomes isolated from the cell juice had as much as ten times the specific radioactivity of 51S particles isolated from dissociated large



Indicates protein & Indicates RNA CA Indicates blockage by chloramphenicol

Fig. 1. Model of biosynthesis of ribosomes.

particles. Thus, the distribution of particle classes has biological significance and is not merely an artifact caused by dissociation. Furthermore, the progressively lower specific radioactivity found in the larger particles indicates a process wherein the large particles are formed from small ones. This is quite likely in view of their similarity in composition. When P³²-labeled cells were subsequently grown in P³¹O₄⁼, the P³² was not accumulated in the large particles but was distributed throughout all classes. This finding indicates a rapid interchange between the small and large particles which may be a consequence of their role in the synthesis of other proteins.

These various facts concerning the biosynthesis of the ribosomes can be summarized in a diagram which should not, however, be taken as a proven model (Fig. 1).

CONTROL OF SYNTHESIS

In a growing microorganism there is usually a balance among the synthetic activities with the result that each constituent has the same rate of formation and the composition changes little from one generation to the next. Balance is also achieved in the synthesis of the smaller molecules so

that each is produced at the rate needed. Disturbances in the environment are frequently accommodated by changes in synthetic rates and after a short transitional period the organism is adjusted to the new situation. Three mechanisms are now recognized which contribute towards this stability. One is the induction of the synthesis of new enzymes; second, is the inhibition of the action of enzymes; and, third, is the inhibition of synthesis of enzymes. Substrate molecules are the most likely to cause these effects but related molecules are frequently active. The relationship of microbial enzyme induction and inhibition to development processes of higher organisms was discussed at a symposium on "The Chemical Basis of Development" (145 to 150).

Umbarger & Brown described the inhibition by isoleucine of threonine deaminase, the initial enzyme in the sequence leading from threonine to isoleucine. Isoleucine decreased by half the requirement of an $E.\ coli$ mutant for threonine. Furthermore, isoleucine inhibited the accumulation of its own precursor α -keto- β -methylvalerate. The affinity of isoleucine for the enzyme was high compared to that of threonine. The advantages of such a rapidly acting feedback system were pointed out. In addition, isoleucine appeared to have some control on the rate of synthesis of the enzyme itself (151).

The control of threonine synthesis was studied by Wormser & Pardee. In extracts of $E.\ coli$ and yeast threonine inhibited the formation of homoserinephosphate and its subsequent conversion to threonine. Threonine did not, however, inhibit the formation of the enzymes in growing cells (152). Similar mechanisms controlling the synthesis of purine nucleotides were

discussed by Magasanik (150).

The control of the formation of an enzyme by one of the products of its activity was demonstrated in a striking way by Gorini & Maas (148). In the presence of arginine, the synthesis of ornithine transcarbamylase was suppressed and the enzyme level dropped to 1 per cent of the usual content. On transferring the cells to an arginine-free medium the enzyme content rose rapidly and then fell. The drop in enzyme level was attributed to inhibition of synthesis by accumulation of endogenous arginine. Confirmation of this interpretation was obtained by growing an arginine-and histidine-requiring mutant in a chemostat with growth limited alternatively by arginine or histidine. It was concluded that the enzyme-forming capacity was twenty-five times greater than was actually used by the wild type cell [see also (149)].

The controls described above are of great advantage to the efficiency of the cell as substrates and enzymes are made only in the quantity needed for balanced growth. An example of a reversed control seems to occur in observations of Sheinin (153). A mutant which would not grow initially in a medium lacking uracil acquired the ability after growth in a uracil-enriched medium. The ability was subsequently lost as the cells in the uracil medium passed through exponential growth into the resting phase. Examination of the enzyme content of the cells showed large increases in enzyme activities

as the cells went from lag to exponential phase followed by a decrease in the resting phase. The five enzymes measured (dihydro-orotic acid dehydrogenase; ureidosuccinic acid synthetase; dihydro-orotase; aspartase and histidase) all showed the same general rise and fall but dihydro-orotic acid dehydrogenase was the most variable. It was not detectable during the initial period when the cells were unable to grow without added uracil. The same enzymes of the wild-type cells showed similar variations through the growth cycle but the dihydro-orotic acid dehydrogenase level never fell to zero. Also the enzyme levels of the wild type were higher in the presence of uracil (153).

This type of positive feedback control leads to instability and would have a marked disadvantage to the cell. It might, therefore, be expected to be less common than the advantageous negative feedback control. A number of the nutritional requirements of mutants may arise from this type of mechanism.

Pardee, Jacob & Monod give further information on the control of enzyme synthesis in studies of the mechanism of enzyme induction. Crosses were made between z+i+ wild-type E. coli which had the capacity to form β-galactosidase but required an inducer and the z-i- mutant which lacked both the capacity to form the enzyme and the requirement for the inducer. Neither parental type could form enzyme without inducer as one lacked the ability and the other required the inducer. Additional markers (T6-sensitivity and streptomycin sensitivity) were used to follow the course of mating and to eliminate the unwanted parents from the population. No synthesis of enzyme was observed after the cross ♂z-i-× 2z+i+ but synthesis commenced within 4 min. after the z⁺ gene entered the cytoplasm in the cross o^{*}z⁺i⁺× ♀z⁻i⁻. After 2 hr., further synthesis of enzyme required addition of the inducer. From these results the authors concluded that the inducible strain carried a repressor of synthesis which was displaced by the inducer. The cessation of synthesis after 2 hr., which occurred in the absence of inducer, was ascribed to the accumulation of the repressor whose synthesis was initiated by the entry of the i+ gene (154).

Other studies of enzyme induction showed that in yeast three enzymes in the sequence galactose \rightarrow uridine diphosphogalactose were independently induced by galactose. Sequential induction by products appeared improbable (155). In *E. coli*, the formation of arginine decarboxylase was stimulated by the addition of arginine, methionine, tyrosine, asparagine, and Fe⁺⁺⁺ to the medium (156). Induction of hydrogenase by *Hydrogenomonas facilis* required incubation in H₂+O₂. The hydrogenase system could develop in the presence of acetate or lactate provided ammonium sulfate was also present (157). The formation of isocitratase in *Pseudomonas ovalis* was maximal during growth on acetate; the enzyme was not formed as long as succinate was present in a mixture of acetate and succinate. Induction may follow the fall in concentration of tricarboxylic acid cycle intermediates which act as repressors (158). In yeast, the formation of succinic dehydrogenase was most

rapid with cells which had reached the end of the growth period. It was suggested that a lipide deficiency might reduce the rate of eznyme formation as supplementing the medium with ergosterol and oleic acid increased the rate of synthesis (159). The inducers, substrates, and inhibitors of an amino acid decarboxylase were studied (160). Biotin-deficient Streptococcus lactis lacked ornithine transcarbamylase. The enzyme activity was restored by incubation with glucose, amino acids, glutamine, and biotin. The reactivation was inhibited by analogues of purines and pyrimidines as well as analogues of amino acids (161).

SYNTHESIS IN DISTURBED SYSTEMS CHEMICAL DISTURBANCES

Several antibiotics are now known to cause different degrees of inhibition of synthesis of the major macromolecular constituents of microorganisms (96). A four-year-old study recently published, showed that chloramphenicol caused a prompt inhibition of protein synthesis in E. coli, but that nucleic acid formation continued at an unchanged rate. However, whereas in control cultures the nucleic acid increased exponentially, in antibiotic-treated ones it increased linearly. This change was accompanied by excretion into the medium of purines and pyrimidines, including guanine, hypoxanthine, and uracil (162). The tetracyclines also have a much greater effect on protein than on nucleic acid synthesis. In E. coli it has been found that the effects of subbacteriostatic concentrations of chloramphenicol and tetracyclines were additive; the authors concluded that chlortetracycline and oxytetracycline act at the same locus but that chloramphenicol inhibits a different step in protein synthesis (163). C14-glycine incorporation by Azotobacter agilis was used to study RNA, DNA, and protein synthesis. All three processes were equally sensitive to inhibition by Co and UO2++ but chloramphenical inhibited protein most and RNA synthesis the least (164). The data do not justify the claim that the formation of DNA was as sensitive as that of protein. The kinetics of glycine incorporation exhibited curious properties which have not been explained.

The antifungal agent, actidione (cycloheximide) also selectively inhibited protein and DNA synthesis (almost complete inhibition at 0.5 to 1.0 μ g./ml., the growth inhibitory concentration for the test organism, Saccharomyces carlsbergensis), whereas RNA synthesis was much less sensitive (165).

It is thus possible to enrich microorganisms with respect to their RNA content and much interest centers around whether or not such additional RNA is supernumerary, functional, unstable, and so on.

Pardee et al. showed that RNA produced by E. coli in the presence of chloramphenical had different physical properties from those of "normal" RNA (electrophoretic mobility, sedimentation rate, ease of dissociation from protein) (166). Neidhardt & Gros, and Hahn et al. then found that when the drug was removed this newly formed RNA was degraded and excreted (167, 168). It has also been reported that RNA formed in the presence of

chloramphenicol breaks down continuously both before and after the removal, unlike the stable RNA formed prior to the addition of antibiotic (169).

Gros & Gros reported the full details of their investigation of the role of amino acids in nucleic acid synthesis. It has been known for some time that amino acid deficiencies often inhibited nucleic acid synthesis. This inhibition did not seem to be a secondary result of the inhibition of protein synthesis as it was also known that nucleic acid synthesis continued when protein synthesis was blocked by chloramphenicol. A series of amino acid-requiring mutants of E. coli (methionine, tryptophan, phenylalanine, threonine, proline) were all found to require the presence of the appropriate amino acid for both RNA and DNA synthesis. Presence of chloramphenical (12.5 to 40 μg ./ml.) caused a slight increase in the nucleic acid synthesis but the addition of the required amino acid caused a further striking increase. Analogues of the required amino acids were also effective in stimulating the synthesis of nucleic acids. The increase in the RNA synthesized compared to the quantity of amino acid added led the authors to suggest a catalytic role (170).

The requirement for amino acids is not universal. Several years ago a methionine-requiring mutant of E. coli was found to continue nucleic acid synthesis in the absence of methionine. Further work on this mutant showed that RNA which accumulated (80 per cent increase) during methionine starvation was not degraded after methionine was added back to the culture. There was, however, a lag before protein synthesis was resumed (171). Aronson & Spiegelman studied the effects of high and low levels of chloramphenicol on the RNA and protein of E. coli. At high levels (500 µg./ml.) RNA synthesis did not require the presence of free amino acids: at low levels (20 µg./ml.) the residual amino acid incorporation occurred into the particle fraction which contained the bulk of the RNA. Addition of C14amino acids to cells which had accumulated RNA in the presence of chloramphenical could result in C14-incorporation into particle-bound protein without concomitant conversion of labeled precursors to RNA. The authors concluded that chloramphenicol allows abnormal accumulation of a normal kind of RNA but prevents its conversion to stable ribonucleoprotein [(172); see also (79)].

RNA levels of E. coli have also been altered by adding chlortetracycline (Aureomycin) or by withholding methionine from a particular mutant. Subsequently, such cells and control cells had the same incremental ratio for newly formed protein to RNA, suggesting that these syntheses were not a function of the total RNA of the cells. Similarly, the inducible β -galactosidase formed the same proportion of the total increase in mass whether control cells were used or cells which had been pregrown in the presence of antibiotic

with or without inducer (173).

The selectivity of a synthesizing system is measured by its discrimination between similar molecules. In microorganisms several steps occur including entry into the cell, activation, and polymerization. As each of these processes eic

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has some degree of specificity, most of the experiments performed to date do not measure directly the selectivity of the template. They do, however, provide qualitative information on selectivity and show the degree of precision in duplication required for biological activity.

The natural amino acid valine could be incorporated to excess by a valine-sensitive strain of $E.\ coli.$ As a result, some of the enzymes formed were inactive and linear growth was observed. β -Galactosidase in particular was not synthesized in an active form (174). In contrast, a methionine-requiring mutant of $B.\ subtilis$ could grow when supplied with L-methionine and DL-ethionine. α -Amylase isolated from these cultures contained 2 methionine/1 ethionine; it had a normal sulfur content, normal electrophoretic movement, and the same activity as usual (175). In this particular case, ethionine acted like selenomethionine which can completely replace methionine without loss of any essential biological activity (176). More commonly, however, ethionine caused inhibitions. $Torulopsis\ utilis$ grown with ethionine formed S-adenosyl ethionine which transfers ethyl groups. Part of the inhibitory effect of ethionine may be caused by this (177). The formation of the natural compound, S-adenosyl methionine, by a yeast enzyme was described (178).

Partial replacement of an amino acid by its analogue often leaves some doubt whether the analogue caused the formation of new proteins, or whether it caused uniform or nonuniform replacement in all proteins. Chromatography of the proteins of *E. coli* after growth on C¹⁴-glucose, S³⁵-methionine, C¹⁴-phenylalanine, 1-C¹⁴-norleucine, or 3-C¹⁴-p-fluorophenylalanine showed quite similar patterns, indicating that the analogues caused no major change in the proteins and that they entered a wide range of different proteins. Eight well-resolved protein fractions which could be readily identified were selected and the degree of replacement of methionine by norleucine was found to be the same in each. Thus, the sites of methionine incorporation in different protein-forming systems all seem to have an equal probability of analogue substitution (79).

Tests of ten analogues were reported by Pardee & Prestidge. DL-7-Azatryptophan was found to be incorporated into proteins of *E. coli* but active enzymes and infective bacteriophage were not produced (179). Incorporation of *p*-fluorophenylalanine was observed in *Lactobacillus arabinosus* protein (180).

Mutants of *E. coli* resistant to analogues have been isolated. Their decreased sensitivity resulted from secretion of large quantities of the natural compound (181). Thus, a mutant resistant to *p*-fluorophenylalanine excreted a substance suspected to be tyrosine which interfered with incorporation of the analogue (182).

The effects of p-fluorophenylalanine on yeast have been re-examined because of earlier contradictory results. The analogue caused a change from exponential to linear growth rate but did not affect the differential rate of protein to nucleic acid synthesis. Cell division was inhibited but mass in-

creased about fourfold before growth ceased. As had been found by others, p-fluorophenylalanine was incorporated into protein (up to 12 per cent of the normal phenylalanine content in growing and 61 per cent in "resting" cells). The analogue did not inhibit either protein synthesis or utilization of the amino acid pool; the high pool levels observed in its presence were attributed to an increased rate of protein breakdown and the contribution of

the p-fluorophenylalanine itself (183).

In studies of E. coli it was found that β -2-thienylalanine (1 μ g./ml.) inhibited protein and RNA synthesis by 85 per cent but DNA synthesis by only 35 per cent so that organisms could become enriched with respect to DNA. This did not increase the rate of RNA or protein synthesis or of inducible enzyme formation but cells became less ultraviolet-sensitive (184). Growth of mutants of Neurospora in minimal medium led to death of inositolless strains but not of leucineless strains. The former were able to make protein and nucleic acid, whereas the latter were not and the authors concluded that the resulting unbalanced growth in the former case was the cause of death (185). The effects of lack of deoxyribosides, uracil, and amino acids in Lactobacillus acidophilus (which requires all of these) have been measured. Deficiency of the first impaired DNA formation almost completely but had little effect on RNA or protein, whereas omission of uracil or amino acids markedly reduced RNA and protein synthesis while permitting some or much DNA formation (186). The same workers noted that the deoxyriboside derivatives of the acid-soluble fraction were not free nucleosides or nucleotides. They became growth-supporting for deoxyriboside-requiring lactobacilli only after digestion with crude snake venom. In media deficient in deoxyribonucleosides, the pool decreased as DNA synthesis occurred (187). 5-Fluorouracil was claimed to allow protein synthesis in a uracil-less strain of E. coli although RNA increased at a much slower rate and DNA decreased (188). Another report suggested that this analogue affected uracil metabolism causing growth inhibition but not death, whereas 5-fluorouracil deoxyriboside was converted to the nucleotide which irreversibly inhibited thymidylate synthesis, causing thymine deficiency, unbalanced growth, and death (189). Barner & Cohen have also studied a uracil-less mutant of the thymine-requiring E. coli 15_T. In the absence of thymine, DNA was not made whether or not uracil was present. Conversely, RNA was not made when uracil was absent whether or not thymine was added. Protein synthesis, however, continued unimpaired for 60 min, in the absence of either or both thymine and uracil. Under these conditions there was some turnover of RNA (190).

When 6-azauracil was added to E. coli growing in synthetic medium, it was converted to the riboside and this, together with uracil, orotic acid, and hypoxanthine, was found in the cell extract and the medium. It was suggested that blockage of nucleic acid synthesis by the analogue resulted in accumulation of precursors (191). E. coli incorporated 5-bromouracil but not 5-nitrouracil or 5-methylcytosine. Replacement of thymine by 5-bro-

mouracil was very much greater (up to 48 per cent) in a thymine-requiring strain than in the wild type (4 per cent) (192).

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Bacillus cereus, partially inhibited by 8-azaguanine, incorporated this analogue into RNA mainly at the ends of chains and in place of guanine. It was released again on adding guanine to the culture (193). In another study with B. cereus, a low concentration of 8-azaguanine was used and it was found that incorporation proceeded linearly for 60 min., reaching a maximum at 120 min, and then being lost from the RNA. After the first 15 min., the mean generation time almost tripled and protein synthesis was strongly inhibited. Synthesis of DNA and cell wall were unaffected but RNA formation was at first faster but afterwards about normal in rate. The RNA synthesized in the presence of the drug was abnormal in containing 8-azaguanine and in being more readily hydrolyzed by acid (194). The same analogue was shown to inhibit formation of the extracellular lysozyme of B. subtilis (195). On the other hand, another study indicated that the analogue reduced incorporation of S35-methionine (and S35-cysteine or S35O4=), resulting in synthesis of abnormal protein. It was suggested that 8-azaguanine might act at the coenzyme level (196).

Nitrogen mustards have been shown to react with nucleic acids (197, 198) and Harold & Ziporin have studied the effects of nitrogen and sulfur mustards on nucleic acid and protein formation by E. coli. These reagents stopped cell division, gave a transient inhibition of DNA synthesis accompanied by accumulation of deoxyribose in the cells, but RNA and protein synthesis were unaffected. The consequences were in many ways similar to those of ultraviolet-irradiation but there is still no explanation of the lethal effect. Conditions determining the resumption of DNA synthesis after removal of sulfur mustard have been investigated. Provision of N, C, and energy sources was necessary although a thymineless strain did not require thymine. It seemed from these and from studies with inhibitors that synthesis of both protein and RNA, but not DNA, was a prerequisite for restoration of DNA formation (199, 200).

Two nitrogen mustards were found to inhibit protein synthesis 100 per cent and DNA synthesis 50 to 100 per cent under conditions which did not affect RNA formation by Aspergillus nidulans. The antibiotic, gliotoxin, however, eliminated protein and RNA synthesis without reducing DNA (201). Then, again, ethidium bromide inhibited synthesis of DNA by the flagellate, Strigomonas oncopelli, while that of protein and RNA continued for some hours (202). Newton also studied the action of antrycide on the same organism. Exponential growth in glucose/peptone medium was converted to linear growth by 10 µg./ml. and protein, RNA, and DNA formation were influenced in the same manner. There was, however, no effect on net synthesis of RNA or DNA by washed cell suspensions nor on C¹⁴-uracil and C¹⁴-glycine incorporation. The fixation of C¹⁴-adenine and C¹⁴-guanine, in contrast, was reduced to 80 to 90 per cent almost at once (203).

Certain unsaturated fatty acids inhibit growth of many species of Gram-

positive bacteria. The effect of linoleic acid (2µg./ml.) on Lactobacillus casei could be annulled by an equimolar concentration of vitamin D2 but not by other steroids such as cholesterol. The nature of the inhibition and reversal has been studied by McQuillen and Kodicek. They found that although linoleic acid caused lysis of isolated protoplasts of B. megaterium and although this could be specifically antagonized by vitamin D2, it seemed unlikely that bacteriostasis could result from simple interference with the cytoplasmic membrane. Kinetic studies with radioactive tracers showed that energy-requiring entry of amino acids and purines and pyrimidines into the pool was unimpaired or even enhanced by concentrations of linoleic acid which caused major inhibition of incorporation of these substrates into protein and nucleic acid. Vitamin D₂ restored the system to normal. It was suggested that linoleic acid might act at a locus near the cytoplasmic membrane, preventing substrates which had passed across the membrane from entering the subsequent synthetic pathways but not from entering the pool (204).

Additional reports on synthesis and mode of action of analogues are appened: Diamino acid analogues (205); amino acid antagonisms in *Tetrahymena* (206); an isoleucine antagonist in *E. coli*, 3-cyclohexene-1-glycine (207); carbamyl-serine, azaserine and S-carbamyl cysteine compared with respect to reversal by glutamine and effect on ornithine/citrulline conversion (208); 3-deazafolic acid antagonized folic acid and 1-deazaguanine antagonized guanine (209). Inhibition of *Lactobacillus arabinosus* by pteridines was augmented by Kinetin (210). A-methopterin blocked *de novo* purine synthesis in *E. coli* (211); aminopterin caused accumulation of alanine and valine in *Aerobacter aerogenes* (212).

Even in cells grown without the addition of analogues unsuspected nucleic acid constituents appear. In addition to the bases adenine, guanine, cytosine, and thymine, DNA's from various sources have been known for some time to contain 5-methyl cytosine and 5-hydroxymethyl cytosine. Recently, Dunn & Smith identified 6-methylamino purine in DNA from E. coli, A. aerogenes, M. tuberculosis, pneumococci, and from E. coli phage T2 and Salmonella-c phage. It was not found in S. aureus, B. cereus, or Streptomyces griseus phage. Normal levels equal to 2 per cent of the adenine content occurred in some bacterial DNA's (e.g., E. coli B/r, K 12, and 15_{T-}) but growth of E. coli 15_{T-} in the presence of 5-aminouracil or 2-thiothymine, or in a low concentration of thymine, resulted in DNA containing 6-methylamino purine equivalent to 15 per cent or more of the adenine (213).

Additional bases have also been reported in RNA. Thymine, 2-methyl adenine, 6-methylamino purine, and 6-dimethylamino purine (214, 215) and 1-methyl guanine, 2-methylamino-6-hydroxy purine, and 2 dimethylamino-6-hydroxy purine (216) have been found in most of the bacterial and plant RNA's. Usually amounts less than one per cent of the uracil content were present. Amos & Korn reported that RNA from E. coli K 12 contained 1 to 2 per cent of 5-methyl cytosine (217).

CELL WALL SYNTHESIS AND ITS INHIBITION

Some aspects of biosynthesis and interference with biosynthesis of bacterial cell wall components have been reviewed (96, 204) and many new studies have appeared. The chemical constitution (but not yet the intimate architecture) of the cell walls of some Gram-positive bacteria is being elucidated but less is known about the more complex envelopes of Gram-negatives. It has been demonstrated, however, in ultrathin sections of *E. coli* that there is a triple-layered wall and a separate cytoplasmic membrane (218).

A completely new class of macromolecules, the teichoic acids, was described by Baddiley and his colleagues (219, 220, 221). They have been extracted from cells and isolated cell walls of some Gram-positive bacteria by cold TCA and consist of polymerized ribitol phosphate with hexose or N-acyl-hexosamine attached to some or all of the ribitols and with alanine esterfied to the sugar. Cytidine diphosphate ribitol appears to be the precursor of the polyribitol phosphate. Cytidine diphosphoglycerol and polyglycerol phosphate were also found but the latter could not be obtained from cell wall preparations and these substances may be related to the polyphosphatidic acid reported to be present in protoplast membranes (204, 222).

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Walls from Gram-positive and -negative bacteria contain mucopeptide consisting of N-acetylated amino sugars, p-glutamic acid, L- and p-alanine, diaminopimelic acid (or lysine, or both) with sometimes one or two other amino acids. Polysaccharide may also be present and Gram-negative bacteria contain lipoprotein in addition. Fragments of these components have been isolated from various sources. B. cereus and B. anthracis polysaccharide fractions yielded a peptide containing alanine, glutamic acid, aspartic acid, and diaminopimelic acid (223). Freeze-dried cell walls of Streptococcus faecalis and Pseudomonas sp., on extraction with cold water or cold TCA, gave nondialyzable material containing cell wall sugars, hexosamine, and traces of amino acids-this did not occur if wall preparations were not first dried (224). A polymer, colominic acid, was isolated from the culture medium of E. coli and shown to have units of N-acetylneuraminic acid (O-sialic acid), a substance formally related to N-acetylmuramic acid which occurs in the cell wall of E. coli (225). Also separated was a substance having colicine activity. Purified preparations of this material, like the O-antigen of this organism, contained protein and lipocarbohydrate. On dissociation of both colicine and O-antigen, inactive lipocarbohydrate and protein with colicine activity were obtained (226). A lysine-requiring strain of E. coli, when grown with limiting amounts of lysine, excreted lipomucoprotein into the medium. This contained diaminopimelic acid and could be fractionated with 90 per cent phenol to give the phenol-soluble fraction which contained glucosamine, ethanolamine, and amino acids (but not sugars, muramic acid, or diaminopimelic acid) and the phenol insoluble fraction which contained aspartic and glutamic acids, lysine, alanine, glucose, and galactose but no hexosamine. Apart from the absence of muramic acid the latter fraction resembles the phenol-insoluble part of the cell wall of E. coli and it may be that when lysine is limiting, incomplete cell wall material is excreted into the medium (227).

Polysaccharides which may be related to cell walls have been found in the culture medium of *Rhizobium* (228) and *Aerobacter aerogenes* (229). The former hydrolyzed to give glucose, galactose, mannose, and unknown components; the latter yielded nearly 30 per cent glucuronic acid together with glucose, mannose, and rhamnose. A chloroform-soluble lipopeptide(s) has been prepared from the alcohol-ether extract of *Nocardia asteroides*. Phosphorus, halogens, glycerol, sugars, and inositol were absent but threonine, alanine, valine, isoleucine, leucine, and proline were detected and the lipide moiety represented about 30 per cent by weight (230). It is not known whether the material is related to wall or membrane.

Studies of precursors to and enzymes concerned in synthesis of bacterial polysaccharides have continued. Extracts of pneumococci able to convert UDP-glucose to UDP-glucuronic acid were described (231) and a strain of A. aerogenes, which makes a polysaccharide containing fucose, was shown to contain GDP-fucose (232) and an enzyme system which converts GDP-mannose to this substance (233).

The glutamyl polypeptides elaborated by *B. subtilis* have been shown to be either poly-L-glutamic acid or poly-D-glutamic acid or a mixture of these; no mixed polymers occurred (234). The absolute and relative amounts of the two kinds of polymer were found to be a function of the ionic composition of the medium. A concentration of 1.5×10^{-7} M Mn⁺⁺ was optimal for growth while higher concentrations increased considerably the total amount of peptide and the proportion of D-glutamyl polypeptide (235).

Two reports show that cell wall synthesis in *S. aureus* can occur under conditions such that protein synthesis is effectively blocked by chloramphenicol [(236, 237); see also (204)]. A similar situation arises in *S. faecalis* when growth is limited by absence of an amino acid which occurs in the cytoplasmic protein but not in the cell wall (238, 239). Apparently cells from exponentially growing cultures can approximately double their content of cell wall without concomitant formation of protein.

Interference with normal cell wall formation may or may not be rapidly or even ultimately lethal depending on the organism and on the conditions. Many different procedures impair wall formation (204, 240, 241) but the majority of reports deal with the effects of penicillin. This antibiotic allows protein and nucleic acid synthesis to continue but may inhibit a stage in cell wall formation by Gram-positive and Gram-negative bacteria. The probable locus is the synthesis of the mucopeptide which contains muramic acid, hexosamine, and a limited range of amino acids. Growth, or rather protein synthesis, is necessary for the bactericidal action of penicillin (96, 242, 243) but provision of a medium of high osmotic pressure can, under suitable conditions, prevent rapid lysis and lead to the formation of fragile, globular forms (spheroplasts) (204, 241, 244, 245, 246). Two main kinds of observation implicate the mucopeptide in penicillin (and certain other anti-

biotic) action. Substances which inhibit incorporation of amino acids into the relatively simple cell wall structure of S, aureus also cause accumulation of derivatives of uridine S'-pyrophosphate-acetylamino sugars which contain one or more of the cell wall amino acids and which are believed to be precursors of the wall. These inhibitors include penicillin, cycloserine (Oxamycin) bacitracin, glycine (0.6M) and chlortetracycline [(247); see also (204, 248, 249)]. Moreover, the fragile, globular forms of Gram-negative species produced by growth in penicillin media have a lower content of mucopeptide constituents than have the normal forms (204, 246).

There appear to be relationships between isolated protoplasts of Grampositive bacteria in which the cell wall is completely absent, spheroplasts of Gram-negative bacteria in which part or all of the mucopeptide is missing although the major fraction of the wall (lipoprotein) is still present, and bacterial L-forms in which has occurred some modification to the wall structure. Stable L-forms of *Proteus vulgaris* induced by action of penicillin were reported to contain little or no diaminopimelic acid or amino sugars (250, 251) but, curiously enough, the unstable L-forms were claimed to have a greater content of diaminopimelic acid than had the corresponding bacillary forms (251). A study of E. coli indicated no major differences in wall composition whether cultures were grown in the presence or absence of penicillin (252). Unfortunately, there was no investigation of the minor mucopeptide component (perhaps only 20 per cent of the whole) which is believed by some to be affected. It may well be that this antibiotic acts on processes connected with secretion of cross-wall material and so interferes with cell division [see, for example (253)]. Only rarely have spheroplasts been observed to divide but Lark (245) has shown that penicillin-induced globular forms of Alcaligenes faecalis can grow exponentially in tryptone medium although not in simple synthetic medium, which supports the growth of the bacillary form. He also found, by using synchronously dividing cultures, that shortly before division the cells were less readily induced by penicillin to become spherical (254).

The consequences of the total absence of cell wall and the properties of isolated protoplasts have been discussed (255) and the relationship of these to spheroplasts has been reviewed (256, 257). Procedures involving amino acids can also yield spheroplasts. For example, growth of *E. coli* in the presence of glycine or serine (258); growth of a mutant strain of *E. coli* in the absence of its required diaminopimelic acid (259); growth of *A. faecalis* in the presence of any one of several D-amino acids (260). It has also been suggested that 6-azauracil may be converted to a nucleotide derivative which competitively inhibits functioning of a normal uridine-containing cell wall precursor (261).

BACTERIOPHAGE

Bacteriophage systems are of considerable advantage in the study of protein and nucleic acid synthesis since infection leads to more or less exclusive formation of new types of protein and DNA. The requirement for a period when protein synthesis can occur before phage DNA formation becomes independent of protein synthesis has again been demonstrated in the case of phage T₂ with chloramphenicol as the inhibitor of protein synthesis (262). The effect of the same antibiotic on DNA synthesis in all seven T-phage systems has been reinvestigated and the results confirmed that cells infected with T₂, T₄, or T₆ did not make DNA unless the antibiotic was added some time after infection. "Prior protein synthesis" was not obligatory in the case of T₁, T₃, or T₇. An earlier stock of T₅ had behaved like these but has been shown to be spurious; authentic T₅ behaved like the T-even phages in this respect although not in others (263). B. megaterium phage M₄ which, like the T-odd coliphages, does not contain hydroxymethyl cytosine, behaved almost identically with the HMC-containing coliphage T₂ (263). It was suggested that the requirement for "prior protein synthesis" before DNA is made may be connected with the absence of genetic homology between phage and bacterium [cf. Stent (107)].

The DNA synthesized in the presence of chloramphenicol can, on removal of the drug, become incorporated into phage particles, and Tomizawa has shown that ultraviolet-irradiation during formation of such DNA leads, on removal of the chloramphenicol, to suppression of further DNA synthesis. The ultraviolet-damaged DNA is, however, incorporated into phage particles many of which are noninfective. It appears that phage-precursor DNA made in the presence of the antibiotic presents an ultraviolet-sensitive target equivalent in all respects to that of completed phage particles. The lesions produced by irradiation under these conditions persist but are not replicated (264). It was later found by Hersey et al. that genetic recombination could occur between phages in the presence of chloramphenicol (265).

Preliminary reports of the rapid turnover of a small fraction of RNA in E. coli infected with phage (266) have been amplified by Volkin et al. (267, 268). After infection by phage T₂ there was a rapid incorporation of P³²O₄³⁵ into RNA (although no net synthesis) and subsequently most of this P³² was transferred to phage DNA. The RNA was not an obligatory precursor, however, since much more P³² appeared in the DNA than passed through the RNA (267). A similar situation obtained in T₇ infection except that here incorporation and turnover of P³² in RNA occurred faster than phage DNA synthesis (268). In both cases the adenylic acid and uridylic acid of the RNA had higher specific radioactivities than had the other two nucleotides and it was noted that the base ratios of the RNA being formed after infection differed from those of normal host RNA but resembled those for the corresponding bases of the phage DNA.

Experiments with T₂ showed that choramphenical added early inhibited phage protein and DNA synthesis and RNA turnover, but that it allowed some labeling of the RNA: if added 10 min. after infection, RNA turnover and DNA synthesis could both occur. The conclusion that DNA formation might be correlated with RNA turnover rather than synthesis was not shared by Watanabe et al. who found that choramphenical added 10 min.

after infection of *E. coli* with T₂, inhibited liberation of P³² from RNA formed after infection but did not affect DNA synthesis (269). In an induced lysogenic system, thiouracil and azaguanine were found to inhibit phage protein synthesis to a much greater extent than that of the host protein. Reversal of the analogue effects could readily be achieved even after some hours by adding uracil or guanine, respectively (270). Volkin *et al.* (268) and Jeener (270) suggested that infection or induction initiates the formation of a highly specific RNA before phage protein and DNA can be made.

The phage T_5 system exhibits differences from those of the other T-series. Total DNA decreased after infection and this fall could be prevented by chloramphenicol which also stopped the subsequent DNA synthesis (271, 272). The decrease occurred whether or not injection followed adsorption and the total DNA fell to about 30 per cent of the initial in 10 min. Chloramphenicol reduced this loss and inhibited protein synthesis to about the same extent. After the DNA reached a minimum, net synthesis occurred at a rapid linear rate but this was stopped by the addition of chloramphenicol within a few minutes; a period longer, however, than was necessary to inhibit protein synthesis (272).

Treatment of E. coli with "ghosts" of T₂ left N assimilation at 80 per cent of normal and phosphate incorporation into large molecules at about the normal rate. The breakdown of DNA which occurs after infection with

intact phage was not observed with "ghosts" (273).

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Since phage-infected bacteria may have to make DNA at a rate equivalent to that of total nucleic acid synthesis in uninfected cells, two important changes are involved: deoxyribose derivatives have to be made in place of ribose derivatives and much more thymine has to be synthesized. It was found that labeled phosphate given to cells infected with T_2 rapidly appeared in deoxyribonucleotides of the soluble fraction, whereas these substances could scarcely be detected in uninfected bacteria (274). Further, phages T_2 , T_4 , T_6 , and T_5 could confer the ability to synthesize thymine on a thymineless mutant of E. coli B_2 , but T_1 , T_3 , and T_7 could not. This is interesting in view of the much greater rate of de novo synthesis of DNA which occurs in cells infected with the first group. There was also evidence of induction of a new pathway of thymine synthesis by T_5 in cells which had mutated to thymine-independence (B_3R) (275).

Lysogenization of *E. coli* K 12 by phage λ -22 caused cessation of RNA, DNA, and protein synthesis for a period of 60 to 100 min. (276).

PHYSICAL DISTURBANCES

Enucleation experiments to show the role of the nucleus in metabolism were first conducted in 1877 and the technique still continues to yield useful information. A comprehensive description of some experiments and their results was given by Brachet (277) and a more recent summary has appeared (278). Further work this year showed the effects of amino acids, purines, and pyrimidines and nucleosides and their analogues on nucleate and anucleate fragments of *Acetabularia mediterranea*. The analogues were

found to be less inhibitory than were the natural substances, the nucleate fragments being less inhibited than the anucleate ones. Labeled thymine and thymidine were incorporated but most of the radioactivity was found in RNA (279). Growth, protein synthesis, and differentiation were observed in nucleate and anucleate fragments of *Acetabularia*. These processes continued for two weeks in both types of fragments but were completely stopped by treatment with RNase. On removal of the RNase, the nucleate fragments recovered and all three processes returned to normal rates. In contrast, there was no recovery in the enucleated halves (280).

Irradiation is another kind of physical disturbance which can be highly selective in its action. It has long been known that the cell division process is by far the most sensitive to ultraviolet light. Quantitative data on the inhibition of division of *E. coli* were presented by Deering. Survival curves showed nearly complete but temporary inhibition of division at doses which had little effect on colony formation and no effect on the increase of DNA, RNA, or total mass. The action spectrum had a maximum at 2652A and a minimum at 2300A. The inhibition of division could be reversed by visible light (281).

In E. coli a dose of ultraviolet radiation can be chosen which has little effect on growth or RNA synthesis but which produces an inhibition of DNA synthesis lasting 20 to 25 min. Using these conditions, Harold & Ziporin found that during the recovery period a blockage of protein synthesis which could be caused either by lack of phenylalanine in the mutant 15_{T} -PA- or by chloramphenicol, prevented the resumption of DNA synthesis. In contrast, thymine was not required during recovery (282). The authors point out that this requirement for a short period of protein synthesis is similar to the requirement for prior protein synthesis in phage-infected cells. The same effect occurs in mustard-treated bacteria.

Hanawalt used the coli mutant 15_T to observe synthesis during unbalanced growth caused either by thymine deficiency, ultraviolet radiation, or both. Numerous curves were presented which show the incorporation of tracers into DNA, RNA, and protein following various doses of ultraviolet radiation. Experiments were carried out to show the effect of thymidine and reactivation by white light. Linear incorporation into both protein and RNA was observed when DNA synthesis was inhibited by ultraviolet light, and exponential rates of synthesis resumed shortly after DNA synthesis began again. A linear rate of RNA synthesis (but not of protein formation) was obtained when DNA synthesis was blocked by thymine deficiency. These results support the concept that the quantity of intact DNA governs the rate of RNA synthesis whereas the quantity of functional DNA and RNA both govern the rate of protein synthesis (283).

Alper & Gillies, from considerations of various suboptimal growth conditions which permit recovery of colony-forming capacity after ultraviolet irradiation of *E. coli*, concluded that unbalanced growth was the principal cause of lethal action (284).

Kempner & Pollard used γ -rays, deuterons, and α -particles to determine

the radiation targets for incorporation of amino acids and glucose into $E.\ coli.$ Gamma irradiation showed that the sensitive volumes corresponded to spheres of radii 80 to 130 A in rough agreement with the size of ribosomes. For methionine incorporation, the cross section measured with deuterons and α -particles was consistent with a sphere of these dimensions. The situation was quite different for proline; α -particles and deuterons indicated a larger cross section than for methionine and were not consistent with a spherical target (285). These findings are surprising as it would be expected that methionine and proline incorporation would simply provide two equivalent measures of protein synthesis. These experiments were described in greater detail by Kempner (286).

Pollard & Kennedy presented studies of the effect of x-rays on P and S incorporation into E. coli. Linear incorporation rates of both PO_4^m and SO_4^m were observed after doses as low as 14,000 r for PO_4^m and 43,000 r for SO_4^m . The 37 per cent dose for S^m incorporation was twice that observed for SO_4^m , indicating a high radiation sensitivity of the intervening biochemical steps. The low 37 per cent dose observed for PO_4^m incorporation (14,000 r) led the authors to suggest that intact DNA may control PO_4^m uptake (287). Other studies of the phosphorus metabolism of continuously irradiated yeast

were reported by Spoerl & Looney (288).

The effects of internal radiation were reported by McFall, Pardee & Stent. Cells of E. coli, highly labeled with P32, were stored in the cold for various periods to allow decay of the P32 and then assayed for viability and various synthetic capacities. The effective disintegrations were thought to be those in DNA as cells labeled in the RNA (by exposing the mutant 15_{T-} to P³² in the absence of thymine) were less sensitive. Cells of strain B/r which contain more nuclei were also less sensitive. On the other hand, phosphorusstarved cells showed a single hit curve but the same final rate of inactivation after reduction of DNA and RNA to \(\frac{1}{2}\) and \(\frac{1}{6}\) of their usual content. Loss of enzyme-forming capacity showed approximately the same sensitivity as loss of colony-forming capacity, but RNA and protein synthesis continued for about 45 min. Assuming that the primary action is localized in the DNA, the survival curves indicate that a P32 disintegration has a probability of 1:50 of destroying the integrity of the nucleus which seems to be required for colony or inducible enzyme formation. This sensitivity is only slightly less than that of phage where a disintegration has a probability of 1:20 to 1:25 of inactivating the particle (289).

In contrast to this special sensitivity of DNA in *E. coli*, decreased incorporation of C¹⁴-phenylalanine by *Amoeba proteus* was observed whether ultraviolet-irradiated nuclei were transplanted to unirradiated cytoplasm or vice versa (290).

SOME THEORETICAL IMPLICATIONS

It is generally accepted that there are inter-relationships among DNA, RNA, and protein more intimate than those between any of these and other macromolecules [but Wilkinson (291) suggests that heteropolysaccharides

might be an exception]. DNA is widely held to be the ultimate repository of the information necessary to perpetuate the characters of individual organisms. Similarly, RNA is thought to intervene between DNA and protein. The nature of these couplings is still a matter of speculation. Many current theories revolve around the idea that the sequences of bases in DNA is related to that in RNA and that both are in some way responsible for the ordered sequence of amino acids in protein chains. Methods for determining the latter are well developed and procedures for DNA and RNA are being investigated (292, 293).

No immediate correlation between the base composition of DNA and RNA is apparent but it is claimed that in a series of species, as the (G+C)/ (A+T) ratio of the DNA increases, there is a tendency for the (G+C)/ (A+U) ratio of the RNA to increase (294). Zubay, on the other hand, has considered a relation between the DNA and the nuclear RNA with the four RNA nucleotides being coded to the four possible base pairs A-T, T-A, G-C, and C-G, where the pair A-T with adenine in one strand of the double helix is different from T-A where adenine is in the other strand (295, 296, 297). The result might be that an RNA chain could be built on an existing 2-stranded DNA forming a 3-stranded structure comparable to some in vitro complexes (24). Since it is now known that bacterial RNA exists both in ribosomal particles and as soluble RNA, it is not to be expected that base analyses of the whole RNA would necessarily be related to those of the DNA. Moreover, although there probably exists a single copy (or at most two or three) of each specific DNA genetic unit in a cell, there may well be a variable number of corresponding RNA molecules.

The most direct evidence of genetic (DNA) control of amino acid sequence in a protein comes from a study of hemoglobins (298) but it is likely that in bacteria also a single mutation results in a single change in a specific protein. It is not known if RNA mediates such a change. Michelson has proposed a model system involving RNA and aminoacyl nucleotides which might function in making RNA, protein, or both (299).

CONCLUSIONS

The major questions which must be asked in connection with the biosynthesis of macromolecules by bacteria seem to be as follows: (a) to what extent are the syntheses interdependent? (b) where are the synthetic systems localized in the cell? (c) what determines the sequences of units in the macromolecules? (d) what determines the rates at which the various molecular species are synthesized? (e) is the current theory of amino acid activation followed by transfer to souble RNA and thence to microsomal particles before being released as soluble protein applicable to bacteria?

Answers to these questions are being accumulated rapidly; first, by recognition of the various separable systems which operate in the intact cell, second, by learning the detailed mechanisms of separate systems and, finally, by studies of their interactions. A variety of partially autonomous systems

have been segregated and, in some, the detailed mechanisms are quite well understood. Less progress has been made in comprehending the interactions among them because of the close couplings which confuse cause and effect.

Many of the energy-yielding reactions and the biochemical pathways for the synthesis of amino acids and nucleotides are now worked out in detail. A beginning has been made in localizing the enzymes concerned. Oxidative phosphorylation seems to be associated with the cytoplasmic membranes. Most of the enzymes formerly reported in the microsome fraction of the cell appear now to be in contaminating protein or in membrane fragments and not in the ribosomes themselves. Two enzymes, however, are definitely located in the particles. These are RNase, which is exclusively in the structure of the particles, and an amino peptidase which is only partially bound.

Three methods for the control of the rates of reactions in the cell have now been recognized and defined. The mechanism of one (inhibition of enzyme action) is reasonably well understood. The other two (inhibition and induction of enzyme synthesis) seem to be closely related but there is yet no clue as to how the inhibitor or inducer affects the rate of enzyme

synthesis.

The synthesis of the small molecules is quite clearly separable from the synthesis of macromolecules. Many species lack some or even most of the capacity to synthesize small molecules without adverse effect on their growth provided the required precursors are available in the medium. Another distinct system which seems to operate in most cells is perhaps more closely related to synthesis of protein and nucleic acid. This is the system which causes the concentration and accumulation of various materials in the cells. It has a certain degree of specificity and it may thereby be the first of several selecting mechanisms which act in sequence to produce proteins with the proper complement of amino acids. Unfortunately, the present theories of the mechanism of accumulation—transport across a permeability barrier or adsorption within the cell—are not yet capable of providing a quantitative explanation for the entire group of experimental observations.

The interpretation of amino acid incorporation has been made more meaningful by the recent recognition of the distinct character of the cell wall. The distinction between incorporation into wall and other protein is particularly evident when cell wall synthesis is blocked. The resulting protoplast is often competent to carry out all the other biosynthetic activities characteristic of living material. Alternatively, cell wall synthesis can occur without the synthesis of other components. Details of the mechanism of cell

wall synthesis are now rapidly accumulating.

An outstanding advance has been made in studies of nucleic acid synthesis. Polynucleotides of both ribose and deoxyribose can now be synthesized by purified enzymes. The rate of DNA synthesis by the purified enzyme is sufficient to account for the synthesis of DNA in growing cells. Both the RNA and DNA systems seem to need a "primer" molecule. If the primer is shown to act as a template, the enzyme would only be required

to facilitate the replication of existing molecules. Such processes seem to satisfy the biological requirements of precise duplication but it remains to be seen whether the products are "natural" and exhibit biological activity. Concurrently, the process of DNA replication in the living cell has been shown to be in accord with the predictions of the Watson-Crick model.

The distinction between soluble RNA and the RNA associated with particles has become more widely recognized and methods are now available to distinguish the two types. Measurements of RNA synthesis will have more significance when the experiments are designed to permit this distinction. It is quite likely that many of the disturbed systems which allow RNA synthesis may turn out to be producing soluble RNA but not completed ribonucleoprotein particles.

Synthesis of protein has not yet been accomplished in any well-defined system. As soon as the internal structure of the cell is disrupted the rate of synthesis drops by a factor of 10 to 20 even in the most successful preparations, whereas an increase in the rate would be expected if nonessential ingredients were being eliminated. Some features of the process have been clarified, however. In the growing cell, the simultaneous presence of all the amino acids is required before any one can be incorporated into protein at its full rate. Such a requirement is often lacking in preparations of broken cells. It has now been shown that the incorporation of single amino acids may proceed into cell wall constituents, into linkages with RNA, or, at extremely low rates, into protein. Cell-free systems capable of higher rates (through still far below the rate achieved by intact cells) require a full complement of amino acids. The incorporation of single amino acids, even though shown to be in peptide linkage in protein, may prove to be caused by a process which is quite different from protein synthesis as it occurs in the growing cell.

Processes which may be preparatory to protein synthesis have been singled out and are under intense study. There seems to be no doubt that amino acid-activating enzymes are ubiquitous in microorganisms and that transfer of amino acids into a linkage with RNA can occur. However, it has not been demonstrated that these RNA compounds are compulsory intermediates of protein synthesis as the alternative interpretation that they might be formed by competing reactions (readily reversible) has not been excluded. Kinetic studies could demonstrate their role as a normal intermediate but both the initial appearance of radioactivity after adding a tracer and its subsequent decline when the tracer is exhausted or diluted out would need to be observed. It would also be necessary to ensure steadystate conditions in order to avoid changes in the quantity of materials which might act as storage reservoirs. Such changes might easily be confused with the kinetic behavior of a true intermediate. Accumulation of a compound does not necessarily prove that the compound is an intermediate particularly when the accumulation is observed in systems which do not permit synthesis of complete proteins. The transfer of amino acids from aminoacyl adenylates to boiled ribosomes (300) is a clear warning that transfer as such is not proof of any biological significance.

The role of the ribosomes in protein synthesis is still supported only by indirect evidence. The rate of protein synthesis is proportional to the ribosome content. Also, most of the RNA, together with a large share of the protein, is found in the ribosomes. It seems doubtful that such a distribution of material could persist through evolution if the ribosomes had no important function. Preparations composed of purified bacterial ribosomes, however, are much less active than preparations containing membrane fragments although the activities are roughly equivalent to those of animal microsomes. These ribosomal preparations, like the animal preparations, show no requirement for a complete range of amino acids. The subcellular systems which are most effective in amino acid incorporation are membrane fragments to which some RNA and DNA still adheres. Their activity is markedly reduced by treatment with RNase or DNase but it may be restored by the addition of nucleic acid or, in some instances, by an "incorporation factor" which stimulates incorporation of amino acids, purines, and pyrimidines. Ribosomal particles with adherent newly formed protein, destined to become a part of the soluble protein, have not yet been detected in bacterial systems, although this is frequently reported in animal systems. Several explanations for this difference include: (a) The bacterial ribosomes act more rapidly so that there is proportionately less newly formed protein adhering. (b) The association is less stable than in the animal particles. (c) The majority of the ribosomal particles of bacteria are not engaged in synthesis at any one time as they must also take part in an autocatalytic process. Perhaps these varied observations may be reconciled if we assume that bacterial ribosomes are only fuctional in protein synthesis when associated with the cytoplasmic membrane.

The interactions of protein, DNA, and RNA remain obscure. Various disturbances to the cell have been studied to find ways of stopping one or another system but usually there is such a strong coupling that the whole system comes to a halt. Protein synthesis may be stopped by chloramphenical without immediate affects on the rates of total RNA and DNA synthesis but the RNA made under those conditions is not in the form of completed ribosomes and may be nonfunctional. Active viral DNA on the other hand can be formed. The synthesis of enzymically active proteins can be stopped by the substitution of amino acid analogues. Growth and nucleic acid synthesis continue at rates limited by the quantity of active enzymes initially present but later stop as the inactive material accumulates. Interference with RNA synthesis, either by analogues or by nutritional deficiencies, often has quite an immediate effect on protein synthesis. Not only RNA but RNA synthesis may be required for protein synthesis. Experiments on the role of DNA give quite contradictory results. DNA synthesis is surely not required; irradiation or thymine deficiency can cause a cessation of DNA synthesis with little effect on RNA and protein synthesis. Enucleation experiments with *Acetabularia* indicate that the RNA-protein synthesizing system can continue but cannot be initiated without the nucleus. Disrupted bacterial cells containing only shreds of DNA can continue to synthesize RNA and protein although at a reduced rate.

In intact bacterial cells, however, the DNA seems to act as a unit. It segregates as a unit and radiation experiments indicate that the integrity of the nucleus (which can be destroyed by a single hit) is essential for synthetic capacities as well as colony formation. Furthermore, sudden increases in osmotic pressure cause temporary contraction of the nucleus and a concomitant cessation of synthesis. These effects are quite unforseen as only a small segment of the entire DNA complement of a cell should be needed to direct the synthesis of any RNA molecule. It may be significant that the disrupted cells are highly exacting in their nutritional requirements. Perhaps the integrity of the nucleus is not essential to its role in RNA synthesis but rather in the organization of the cell for the efficient synthesis of smaller molecules.

The formation of macromolecules of the kind we have been discussing must be a complicated process and in the intact cell the complexity is increased because a wide variety of syntheses is occurring simultaneously. However, it is still necessary to carry out investigations at all levels from purified enzyme systems to growing cell populations.

The ideal will be achieved when there is demonstration of the synthesis of a single, specific protein of known amino acid sequence mediated by a single specific RNA of known nucleotide sequence plus purified enzymes and cofactors and under the ultimate control of a single specific DNA of known nucleotide sequence. This is a goal which at present seems still below the horizon but on considering the advances made in the past few years it may well be reached much earlier than now seems possible.

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ASPECTS OF TERMINAL RESPIRATION IN MICROORGANISMS^{1,2}

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INTRODUCTION

Although oxidative processes in microorganisms have been studied for many years—Quastel (185), in 1925, recorded over 50 different dehydrogenation reactions in *Escherichia coli*—the sequence of reactions whereby substrates are oxidized in carbon dioxide and water remained a subject of controversy until about 10 years ago. Earlier evidence was conflicting because some of the procedures which had established the occurrence of the TCA cycle in animal tissues (134, 136), gave negative results with microorganisms. Such negative results can be satisfactorily attributed to the existence of permeability barriers and it is now accepted that the TCA cycle is the chief pathway of terminal respiration of most microorganisms.

It is the main purpose of this review to discuss some of the difficulties encountered in the study of microbial oxidative processes, to survey some of the evidence in support of the TCA cycle, and to review reactions ancillary to the cycle, including the possible routes whereby microorganisms utilize as substrates compounds more highly oxidized than acetate, such as glycollate, glycine, oxalate, and formate.

DIFFICULTIES IN STUDYING OXIDATIONS IN MICROORGANISMS

Attempts to demonstrate the occurrence of the TCA cycle in microorganisms by the procedures used with animal tissues (134) were frustrated by the existence of barriers preventing the free access of externally added substrates to intracellular enzyme systems. Although most microorganisms readily oxidized pyruvate or acetate, some intermediates of the TCA cycle were oxidized slowly or only after prolonged lag periods. This failure of added materials to stimulate respiration was caused by their nonpenetration into cells. Barron, Ardao & Hearon (21) and Altenbern & Housewright (12), for example, found that the oxidation of succinate by Corynebacterium creatino-

¹ The survey of the literature pertaining to this review was concluded in November, 1958.

² The following abbreviations will be used: ATP (adenosine triphosphate); CoA and CoA·SH (coenzyme A); DPN and DPNH (oxidized and reduced diphosphopyrindine nucleotides); IDP and ITP (inosine di- and tri-phosphates); TCA (tricarboxylic acid); TPN and TPNH (oxidized and reduced triphosphopyridine nucleotides); TPP (thiamin pyrophosphate).

³ I wish to record my indebtedness to Prof. Sir Hans Krebs, F. R. S., and to Dr. S. R. Elsden for their helpful criticisms and advice.

vorans and Brucella abortus was considerably more rapid in acidic solutions than at neutral pH, which suggested that the undissociated forms of the acid penetrated cell membranes more readily than the succinate ion. But even under acidic conditions, the stimulation of respiration resulting from addition of succinic acid was too low for this compound to be considered as an intermediate in pyruvate or acetate oxidation. The nonutilization of, e.g., citrate by E. coli (132) and of all TCA cycle intermediates by bakers' yeast (138) could also be ascribed to these permeability difficulties.

Krebs, Gurin & Eggleston (138) sought to overcome these barriers by rapid exposure of bakers' yeast to low temperatures. This technique had been shown by Dixon & Atkins (62) and by Lynen (146) to facilitate the entry of substrates into the cells. But, although added citrate and fumarate were shown to equilibrate rapidly with isocitrate and malate and must, therefore, have penetrated the cells, these compounds did not stimulate the oxygen uptake of dry ice-treated yeast. It is likely, however, that the treated yeast was inhomogeneous (86, 122, 150) and that those cells which had been rendered permeable to citrate, fumarate, and succinate no longer oxidized acetate (122, 150).

The permeability barriers affected also the use of inhibitors of the TCA cycle. One of the most specific of these is malonate which, in animal tissues, has been shown to abolish the catalytic action of TCA cycle intermediates and to lead to the accumulation of succinate (135). The addition of malonate (186) to microorganisms oxidizing acetate caused no inhibition at neutral pH (12, 21), though some was observed under acidic conditions (21) which contrasted with the inhibitory action of malonate noted with cell-free extracts (8, 200). The use of this inhibitor was further restricted by the ability of some organisms (e.g., Pseudomonas) to metabolize malonate (88, 256). Data obtained with other inhibitors provided tentative support for the TCA cycle. But, though it is probable that the inhibition of acetate oxidation caused by the addition of fluoroacetate (25, 83, 102) was caused by the inhibition by fluorocitrate of aconitase (145, 159), this inhibitor might have affected other metabolic reactions. For example, Elsden & Ormerod (68) found that the reduction of CO2 by Rhodospirillum rubrum in the light, with H2 as electron donor, was inhibited by fluoroacetate. Dagley & Walker (52) noted that a suspension of a Vibrio (87) poisoned with fluoroacetate accumulated pyruvate as well as citrate. This argued for a second site of action of the inhibitor as recently confirmed by Callely & Dagley (32), who showed the accumulation of pyruvate to arise from the inhibitory action of fluoromalate, formed from the condensation of fluoroacetate and glyoxylate. Similarly, the inhibitory effect of arsenite (91, 244) indicated the involvement of keto compounds in acetate oxidation but not necessarily that the route of acetate oxidation was the TCA cycle.

Permeability problems were the cause of much of the confusion arising from the early experiments with istotopic tracers. The considerable literature on this topic has been reviewed by Ajl (4, 5) and by Elsden (67). The

early experiments of Ajl and his co-workers (2, 3, 6, 7) and of Karlsson & Barker (106, 107) gave results which were interpreted as excluding the operation of the cycle in *E. coli* and *Azotobacter agile*. The cycle was held to operate in *Micrococcus lysodeikticus* (10) and in *Aerobacter aerogenes* grown on citrate, but not in the latter organism when it was grown on acetate (258). Experiments with dry ice-treated bakers' yeast were also interpreted as ruling out the cycle as the pathway of acetate oxidation in bakers' yeast (138).

These results were obtained from experiments in which unlabelled substances, suspected to be intermediates in the oxidation of a labelled material, were added to suspensions of microorganisms oxidizing this material. At the end of the incubations, the added carriers were recovered and tested for isotope content. An absence of label in the isolated carrier was interpreted as showing that the carrier compound was not an intermediate in the oxidation of the labelled substrate. Mixing between the added compounds and the endogenously produced intermediates was thought be be ensured by mechanical rupture of the cell barriers (freezing) (138), or by the use of "adapted" cells (2, 7, 106, 107, 225). This latter approach was an application of the "multiple simultaneous oxidation" technique of Stanier (213). From his studies on the oxidative degradation of aromatic compounds, Stanier formulated the idea (213) that "cells grown on a given substrate should attack all intermediate compounds in the reaction series, whereas growth on a compound intermediate in the series should cause adaptation to all subsequent members of the series." On this basis, cells capable of completely oxidizing acetate or any intermediate of the cycle via the cycle should contain the enzymes necessary for oxidizing all members of the cycle. Yet even cells "adapted" to metabolize some cycle intermediates by growing on them as carbon sources, did not oxidize all other intermediates.

THE NATURE OF PERMEABILITY BARRIERS

Analysis of the nature of the processes which regulate the entry of exogenous substrates into cells revealed that the conclusions derived from the earlier experiments with "adapted" microorganisms rested on a misapprehension of Stanier's hypothesis (213). The multiple simultaneous oxidation technique could only be applied, as Stanier (214) emphasized, under conditions of free permeability of the cells to all compounds tested. Experiments on yeast (75), Pseudomonas aeruginosa (35), Azotobacter vinelandii (216, 217), Azotobacter agile (194), and E. coli (249) showed that these conditions were not fulfilled. Although whole cells oxidized some added TCA cycle intermediates only after prolonged lags or not at all, extracts of these cells contained all the enzymes required for the rapid and complete oxidation of these compounds. Moreover, Barrett, Larson & Kallio (20) and Kogut & Podoski (110) showed that the adaptive lags were not associated with the formation of intracellular oxidative enzymes, but with the elaboration by the cells of systems catalyzing the transport of substrates from the medium into the cells.

These transport systems had the characteristics of adaptive enzymes: they were elicited in response to specific substrates, and their formation was abolished or arrested by ultraviolet irradiation (20, 110) and agents such as amino acid analogues (20), purine analogues (156), and chloramphenicol (156) known to inhibit enzyme biosynthesis. Recognition of the existence of these transport systems [permeases: for review see Davis (56) and Cohen & Monod (46)] provided a satisfactory explanation for the oxidative behavior of cell suspensions, for the failure of added malonate to affect these oxidations, and for the nonequilibration of added "carriers" with intracellular isotopic intermediates. Furthermore, the assumption that nonoxidation of an added substrate by whole cells indicated the absence of enzymes capable of oxidizing that substrate was shown to be invalid, and with it the premise on which rested much of the earlier work.

THE ESTABLISHMENT OF THE TCA CYCLE IN MICROORGANISMS

Additional evidence that the TCA cycle is the pathway of terminal respiration in microorganisms was obtained with the aid of two techniques other than the demonstration that oxidations by cell-free extracts (20, 35, 75, 110, 194, 216, 217, 249) obey the criteria used for establishing the cycle in animal tissues (134). One of these techniques consisted of analyses of the intracellular isotope content of microorganisms metabolizing a labelled substrate in the absence of added "carriers"; the other utilized auxotrophic mutants.

Krampitz and his co-workers (225, 226) confirmed the findings of Ajl and his colleagues (6, 7) that some unlabelled TCA intermediates added to suspensions of E. coli metabolizing 14C-labelled acetate acquired little or no radioactivity. In particular, in an experiment (226) in which 2-14C acetate of specific activity of 3.86×105 counts/min./mM was oxidized in the presence of unlabelled α-ketoglutarate and succinate, the succinate recovered had a specific activity of 6×104 counts/min./mM, while that of α-ketoglutarate was only 0.12×104. When, however, the experiment was repeated in the absence of unlabelled carriers (226) with large quantities of cells (80 gm.) and the intracellular cycle intermediates were extracted, the specific activities of all intermediates including citrate were found to be of the same order of magnitude. Furthermore, the distribution of isotope within the molecules was as expected from the operation of the cycle, and the specific activities of carbons 5 and 6 of citrate (which are the sources of carbon dioxide released in the TCA cycle), were closely similar to that of the evolved carbon dioxide. Saz & Krampitz (199) performed similar experiments with Micrococcus lysodeikticus. Again, some unlabelled carriers acquired insufficient label from the metabolized 2-14C acetate to support the operation of the TCA cycle, yet the intracellular cycle intermediates were in isotopic equilibrium with each other, with the expired carbon dioxide, and with the recovered acetate. Although whole cells did not oxidize citrate, extracts prepared therefrom rapidly oxidized this compound and, in the presence of arsenite and fumarate, converted acetate almost quantitatively to α -ketoglutarate (200). These results confirmed by Ajl & Wong (9), demonstrated the fallibility of the "carrier" technique and provided conclusive proof of the operation of the TCA cycle in these microorganisms.

The quantitative importance of the cycle as the major route for acetate oxidation was demonstrated by Gilvarg & Davis (80) with mutants of E. coli and Aerobacter aerogenes which lacked the citrate-condensing enzyme but retained the ability to form acetyl coenzyme A from pyruvate or acetate. As a result of this metabolic block, the mutants exhibited (a) a nutritional requirement for glutamate or α -ketoglutarate; (b) virtually complete inability to oxidize acetate (though succinate was readily metabolized); and (c) a considerable decrease in the extent to which glucose was oxidized to carbon dioxide. These findings offer strong evidence that the cycle is the only significant pathway for acetate oxidation in these species, and that the terminal oxidation of glucose occurs via the cycle.

As indicated in Table I, the occurrence of the cycle has been shown to be so widespread as to lead to the tentative conclusion that all microorganisms, capable of oxidizing to completion any substrate the metabolism of which yields an intermediate of the cycle, utilize this oxidative pathway. Organisms in which components of the cycle are absent, such as Acetobacter suboxydans (108, 192, 193), are also incapable of effecting complete oxidation of ethanol. The claim that Acetobacter peroxydans, which can oxidize acetate, is devoid of isocitric dehydrogenase (231) has not been corroborated by Atkinson (14). Data from experiments with inhibitors which have been adduced as evidence against the cycle (15, 141) are susceptible to different interpretations in view of the "permease" concept of control of cell permeability.

Although the TCA cycle was generally accepted as the major pathway of terminal respiration in bacteria, its role in the oxidation of acetate by bakers' yeast was not clear. It was known that the component reactions of the cycle could occur. Lynen (147, 148, 150), Wieland & Sonderhoff (253), Virtanen & Sundman (241), and Weinhouse & Millington (248) had shown that citrate was formed when acetate was oxidized by bakers' yeast, and Novelli & Lipmann (170) demonstrated that this reaction was caused by the condensation of acetyl coenzyme A and oxalacetate. Citrate was converted to α-ketoglutarate (75, 90, 111) by various types of yeast preparations, though not by intact cells. Yeast extracts catalyzed the oxidation of succinate to fumarate (151) and of malate to oxalacetate (151), and contained fumarase (90, 93) and aconitase (75, 90, 170, 248). Isotope data agreed with the assumption that acetate is converted to citrate and succinate (149, 212, 219, 248). However, many of these reactions appeared to be relatively slow; in particular, cells ruptured by being shaken with Ballotini beads (144, 165 to 168) did not oxidize acetate and oxidized added di- and tricarboxylic acids incompletely and at low rates.

Krebs et al. (138), while agreeing that the TCA cycle can occur, had questioned its role as the main path for oxidation of acetate on the basis of results

TABLE I

SOME MICROORGANISMS IN WHICH EVIDENCE FOR THE OCCURRENCE OF THE TCA

CYCLE HAS BEEN OBTAINED

Organism	Reference	Organism	Reference		
Acetobacter aceti	(229)	Mycobacterium phlei	(26)		
Acetobacter ascendens	(230)	Mycobacterium tuberculosis	(158)		
Acetobacter pasteurianum	(109)	Neurospora crassa	(13, 220)		
Acetobacter peroxydans	(14)	Pasteurella pestis	(69)		
Achromobacter sp.	(204)	Penicillium chrysogenum	(83)		
Aerobacter aerogenes	(80)	m (59)			
Agrobacter radiobacter	(235)	Pseudomonas aeruginosa	(35, 156)		
Alcaligenes faecalis	(235)	Pseudomonas fluorescens	(19, 110)		
Allomyces macrogynus	(30)	Pseudomonas ovalis Chester	(124)		
Ashbya gossypii	(157)	Rhodopseudomonas capsulatus	(218)		
Aspergillus niger	(191)	Rhodopseudomonas	(47)		
Azotobacter agile	(194)	Rhodospirillum rubrum	(47, 66)		
Azobacter vinelandii	(11, 216, 217)	Saccharomyces cerevisiae	(251)		
Bacillus cereus	(22)	Saccharomyces drosophilarum	(131)		
Bacillus subtilis	(252)	Serratia marcescens	(254)		
Baker's yeast	(57)	Shigella flexneri	(177)		
Blastocladiella emersonii	(40)	Staphylococcus aureus	(215)		
Brucella abortus	(12)	Streptomyces coelicor	(45)		
Corynebacterium creatinovor	ans (76)	Streptomyces griseus	(79)		
Corynebacterium erythrogen	es (236)	Streptomyces nitrificans	(201)		
Escherichia coli	(80, 195, 226)	Torulopsis utilis	(48)		
Flavobacterium aquatile	(184)	Zygorrhynchus moelleri	(161)		
Micrococcus lysodeikticus	(200)				

obtained with dry ice-treated yeast. Re-examination of these experiments (122) has indicated that the treated yeast probably consisted of a mixture of undamaged cells (which oxidized acetate but no added TCA cycle intermediates) and disrupted cells (in which the succinoxidase system had been rendered partially accessible to added succinate, but which did not oxidize acetate or other TCA cycle intermediates). Dry ice treatment reduced the rate of acetate oxidation to about 12 per cent of that observed with fresh yeast; this value is close to the estimate of Krebs et al. (138) that their material contained about 7 per cent undamaged cells. Although, with treated yeast preparations, no significant incorporation of label from acetate into succinate was observed, it was noted that intracellular succinate was highly labelled when fresh yeast was used.

Two procedures were used for establishing the quantitative role of the TCA cycle in acetate oxidation by bakers' yeast. DeMoss & Swim (57) found, after incubation for 25 min. of a suspension of bakers' yeast with 2-14C acetate, that the isolated intracellular TCA cycle intermediates had equiv-

alent specific activities, and that the distribution of isotope incorporated from acetate (of specific activity 5700 counts/min./ μ mole of C in the methyl group) in citrate and succinate were as follows:

	Compound				Specific Activity (c/min./µmole of C)						
						1	2	3	4	5	6
1 HOOC	2 CH ₂		4 ·CH ₂	5 ·COOH	6 •СООН	67 [39]	1540 [2210]	870 [870]	1540 [870]	67 [95]	95 [95]
1 HOOC	2 -CH ₂	3 ·CH ₂ ·C	4 00H			435	1695	1695	435		

Since the procedure used for the degradation of citrate did not distinguish between carbons 1 and 5, and between 2 and 4, the "ideal" distribution was calculated and is given in brackets.

From these data, it is clear the citrate and succinate were in isotopic equilibrium, and that the evolved carbon dioxide (of specific activity 97 counts/min./ μ mole of carbon) was derived from carbons 5 and 6 of citrate. This provides excellent evidence for the role of the TCA cycle. However, the citrate was presumably formed from the combination of acetate with oxalacetate and, to account for the observed levels of radioactivity in citrate, carbons 2 and 3 of the oxalacetate, formed from the further metabolism of succinate, must have been diluted by an equal amount of nonlabelled carbon, while carbons 1 and 4 were diluted by three times as much. DeMoss & Swim (57) attempt to explain this finding by postulating the de novo formation of oxalacetate by the condensation of acetate with two endogenous unlabelled C_1 units or one endogenous C_2 unit. Since malate and fumarate were found to be in isotopic equilibrium with succinate, this synthesis of oxalacetate would demand the existence of a large reservoir of unlabelled precursors not in free exchange with other cycle intermediates.

A second type of approach was used by Kornberg & Krebs (122). The endogenous respiration of a dilute suspension of bakers' yeast was reduced by shaking the material with buffer at 30°C. for 2 hr., prior to the addition of unlabelled acetate. When a constant rate of acetate oxidation had been reached 1:2-14C acetate was added and samples were taken rapidly. Analysis of the distribution of isotope in these samples by paper chromatography and radioautography showed that, in the earliest samples, isotope from acetate was incorporated only into intermediates of the TCA cycle and into amino acids directly derived therefrom. In one experiment, 14C from acetate was rapidly and linearly incorporated into citrate, succinate, and malate for the first 10 min., corresponding to the formation of 15, 8, and 11 mµmoles, respectively, of these intermediates per mg. dry weight of yeast. After this time, isotope continued to appear in citrate and, to a lesser extent, in malate,

though not in succinate. This latter increase was found to be caused by a continued synthesis of citrate and malate from acetate. The citrate concentration rose within 3 hr. from 25 to 80 m μ moles/mg. dry wt., while that of malate increased from 6 to 16 m μ moles/mg. dry wt.

Oxidation of labelled acetate was also accompanied by the appearance of large amounts of isotope in glutamate and in a polyglucoside material [cf. Eddy (64)]. There was no significant incorporation of isotope into protein. It is likely, therefore, that the incorporations of isotope into glutamate and possibly into part of the polyglucosan, were the result of isotope exchange reactions.

Moses & Syrett (162) showed that the addition of unlabelled substrates stimulated the release of $^{14}\text{CO}_2$ from ^{14}C -labelled bakers' yeast cells, and hence apparently increased the rate of breakdown of intracellular storage materials. These findings would explain the dilutions with unlabelled carbon observed in the experiments of DeMoss & Swim (57), and would obviate the necessity for postulating *de novo* synthesis of oxalacetate from hypothetical stores of C_1 - or C_2 -units.

It can be calculated that, if 1:2-14C acetate enters the TCA cycle, all intermediates will be saturated with isotope after more than 5 turns of the cycle. In the experiment (122) discussed above, 23 mµmoles of acetate (i.e., 46 mµmoles of carbon) were oxidized/min./mg. dry wt. of yeast, but, since the total quantity of oxygen absorbed was only 67 per cent of the theoretically required amount, the quantity of carbon from acetate flowing through the pools of intermediates was $100/67 \times 46 = 68 \text{ m} \mu \text{moles/min./mg. dry wt.}$ of yeast. The size of the rapidly equilibrating "pools" of TCA intermediates was of the order of 40 mµmoles, containing approximately 180 mµmoles of carbon. If the pathway of acetate oxidation involved these "pools," they would be expected to turn over once in approximately 2.6 min. and to be fully saturated with isotope in approximately $5 \times 2.6 = 13$ min. The finding that the rate of isotope incorporation into citrate, succinate, and malate shows a sharp inflection after about 10 min. is therefore consistent with the view that the "pools" are saturated with isotope at this time, and that all the acetate oxidized passes through them. The continual synthesis of citrate, malate, and possibly of polyglucosan demands the occurrence of ancillary reactions whereby these materials can be synthesized from acetate. The nature of these reactions is discussed in the section on the glyoxylate cycle.

THE DUAL ROLE OF THE TCA CYCLE

Krebs et al. (138) suggested that in growing cells the reactions of the TCA cycle may serve not only to provide energy by oxidation of acetate, but also to provide the carbon skeletons of cell constituents. Tracer studies, especially with E. coli (49, 195), Torulopsis utilis (48, 195), Rhodospirillum rubrum (50), and Neurospora crassa (13, 195) have confirmed this suggestion. In particular, 'families' of amino acids have been recognized, the "parent" member of which is directly derived from the TCA cycle. Thus, in their ex-

tensive studies on E. coli, Roberts et al. (195) showed threonine, methionine, lysine, and diaminopimelic acid to be derived from aspartic acid; and arginine, citrulline, ornithine, and proline from glutamic acid. These interrelationships have been more fully reviewed by Davis (55) and Ehrensvärd (65). Since the "parent" members of these families of amino acids are formed by amination of TCA cycle intermediates (for example, glutamic acid is derived from α-ketoglutaric acid, and aspartic acid from oxalacetic or fumaric acids), the synthesis of protein during the growth of microorganisms is accompanied by a continued drainage of carbon skeletons from the TCA cycle. Similarly, the biosynthesis of carbohydrate, nucleic acids, and other cell constituents (other than fat or steroid materials) from noncarbohydrate precursors causes a removal of C4 intermediates from the TCA cycle [for review, see Krebs & Kornberg (137)]. One turn of the TCA cycle results in the oxidation of one molecule of acetyl coenzyme A to two molecules of CO2 and, on balance, the acceptor molecule for the C2-unit entering the cycle (oxalacetate) is re-formed. No net synthesis of carbon materials is possible in the TCA cycle and intermediates drained from the cycle must therefore be replaced by reactions ancillary to it. When glucose, or any substrate the degradation of which gives rise to pyruvate, is the carbon source for growth, this drainage can be made good by CO₂-fixation reactions (41, 44, 70, 153, 251, 252).

Several such reactions have been recognized to play important roles in the formation of TCA cycle intermediates. They occur either with the coupled oxidation of reduced pyridine nucleotides or with utilization of an "energy-rich" bond [for review, see Krebs & Kronberg (137)]. In the former category are the "malic enzyme" (174), catalyzing the reaction:

and the TPN-linked isocitric dehydrogenase (171)

Both enzymes have been shown to occur widely in bacteria (171 to 174). In the second category are the phosphopyruvic carboxylases. Utter and his colleagues (84, 140, 237, 238, 239) obtained purified preparations of chicken liver which were free of "malic enzyme" activity and catalyzed the reaction:

This enzyme (oxalacetic carboxylase) has been found in extracts of autotrophically grown *Hydrogenomonas* by Judis, Koffler & Powelson (100) and tentatively identified in *Propionibacterium shermanii* by Pomerantz (182).

Formation of oxalacetate from phosphopyruvate without the direct intermediation of nucleoside diphosphates is catalyzed by phosphopyruvate carboxylase:

This enzyme has been reported in extracts of a number of microorganisms, including Propionibacterium shermanii (182) and Thiobacillus thiooxidans

(224). The mechanism of the incorporation of isotope from carbon dioxide into oxalacetate by extracts of *Micrococcus lysodeikticus* (89) in the absence of nucleoside triphosphate, observed by McManus (154) is unknown, as also is the route whereby oxalacetate is formed from carbon dioxide and pyruvate by extracts of *E. coli* (104, 105).

The reversible formation of succinate by carboxylation of propionyl coenzyme A has been studied with *Veillonella gazogenes* (*Micrococcus lactilyticus*) (60, 180, 250) and *Propionibaceterium* (58, 99). The observed results are consistent with the formulation

succinate
$$+$$
 ATP + CoA \longrightarrow succinyl CoA \longrightarrow propionyl CoA + CO₂ \qquad v.

The mechanism of this reaction may involve the intermediate formation of a C_1 compound in rapid equilibrium, but not identical, with CO_2 (60, 180, 183, 262). It should be emphasized that reaction v can lead to the net formation of succinate from propionate and carbon dioxide only in aerobic systems, in which the succinate formed can be further metabolized.

THE GLYOXYLATE CYCLE

The dual role of the TCA cycle, in acting both as the main terminal respiratory pathway of microorganisms and as a source of carbon precursors for the synthesis of carbohydrate, protein, and other cell constituents, demands the operation of reactions ancillary to the cycle to replace intermediates drained away as a result of biosynthetic reactions. Such replacements can be accomplished by CO₂ fixation reactions only under conditions where sources of CO₂ acceptor molecules (chiefly pyruvate or phosphopyruvate) are readily available (reactions i to v).

When microorganisms grow on acetate or compounds the degradation of which gives rise to acetate, as the sole carbon source, such CO₂ acceptor molecules are not available, and alternative reactions must occur to form them or TCA cycle intermediates from acetate. It might be envisaged that this could be accomplished by the direct formation of a C₃-unit from acetate and carbon dioxide, or a derivative thereof. Evidence has been reported for the existence of such condensations in Clostridium kluyveri (233), E. coli (1, 195) and in Rhodospirillum rubrum during anaerobic photometabolism of acetate (50). This evidence stems from interpretations of the labelling of compounds formed when substrates differentially labelled with isotopic carbon are metabolized by whole cells. There is no evidence that cell extracts can catalyze the net formation of C₃ compounds by such condensations.

The exchange of the carboxyl group of pyruvate with carbon dioxide has been observed in extracts of *Clostridium butyricum* (255, 257), *C. acidiurici* (196), *A. aerogenes* (221), *E. coli* (221), *Micrococcus pyogenes* (246), and a rumen organism LC (142). Incorporation of a C₁-unit into pyruvate

⁴ See also reviews by Ajl (5) regarding the evolution of our knowledge of acetate metabolism. Ed.

occurs in extracts of E. coli (222, 240) and Micrococcus lactilyticus (169). It could thus be postulated (1, 195) that C4 compounds are synthesized by the successive carboxylation of acetate to give pyruvate or phosphopyruvate and, subsequently, malate. However, although the formation of malate from pyruvate and CO₂ (reaction i) has been demonstrated in many bacteria, the incorporations of isotope from C1-units into pyruvate catalyzed by cell extracts are isotopic exchange reactions; no convincing evidence exists that such reactions lead to the net formation of C3 compounds. Furthermore, the occurrence of such consecutive carboxylation reactions would involve the incorporation from carbon dioxide of at least 30 per cent of the total cellular carbon.

Although experiments (112) on the effect of unlabelled carbon dioxide on the synthesis of labelled protein from labelled acetate by Pseudomonas KBI (110) indicated that a compound in ready equilibrium with carbon dioxide lay on the path from acetate to protein, the quantitative importance of CO2-fixation reactions during growth on acetate was small; only 15 to 17 per cent of the total carbon incorporated by growing cells had passed through stages exchangeable with CO2 (113, 128). Moreover, although cells grown on a medium with 1:2-14C acetate as sole carbon source contained aspartic acid with a higher isotope content in the carboxyl groups than cells grown in a similar medium gassed with unlabelled carbon dioxide (as expected from reactions i, iii, or iv), no such difference of isotope content was detected in the isolated alanine; all three carbon atoms were of identical specific activity, and no carbon from CO2 had therefore been introduced into this C3 compound. If it is assumed that under these conditions the synthesized alanine was in equilibrium with pyruvate, it must be concluded that no formation of pyruvate from acetate and CO2 can have occurred to any quantitatively significant extent, and that the essential net syntheses of TCA cycle intermediates from acetate were not accomplished by CO2-fixation reactions (128).

Studies of the rate of appearance of isotope from labelled acetate in components of the nonprotein fractions of Pseudomonas KBI (114, 115) also showed that while intermediates of the TCA cycle acquired isotope after 3 sec., phosphorylated intermediates became labelled only after relatively long periods of incubation (20 sec.) which argued against the occurrence under these conditions of carbon dioxide-fixing reactions such as operate in photosynthetic and autotrophic organisms (242).

Since the necessary replacement of TCA cycle intermediates drained during growth on acetate was not accomplished by carbon dioxide-fixation processes, and the TCA cycle appeared to be the sole metabolic route of acetate under these conditions (83), alternative routes from acetate to one or more TCA cycle intermediates had to be sought. One such possible route, the direct formation of succinate by oxidative condensation of acetate:

2CH₃COOH - 2H → HOOC · CH₂ · CH₂ · COOH

vi.

had already been suggested by Thunberg in 1920 (232).

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Although this reaction has been assumed to play a role in the metabolism of bacteria (2, 3, 4, 6, 7, 10, 12, 21, 53, 81, 82, 103, 206, 225, 231, 258), yeasts (27, 143, 248, 253), phytoflagellates (92), and molds (70, 72, 73, 74, 164), no convincing evidence has been presented for its occurrence in these or other organisms. Re-evaluation by Sutherland (223) of the experiments of Topper & Stetten (234) indicates that the observed direct formation of labelled acetate from succinate in rabbit liver slices was caused by the presence of trace contaminants in the isotopes used. The postulated formation of succinate from acetate via reaction vi reported by Davies (54), is rendered doubtful by the observation that the heart muscle preparations capable of incorporating label from isotopic acetate into succinate do not produce acetate from succinate, although the reported equilibrium should strongly favor succinate cleavage (Davies, personal communication). Seaman & Naschke (203) and Seaman (202) claimed to have demonstrated the Thunberg reaction (vi) in extracts of Tetrahymena pyriformis. The rates of succinate cleavage observed were extremely low, and the metabolic significance of this reaction is doubtful. Experiments on the biosynthesis of cell constituents from acetate by E. coli have also been interpreted (81, 82) to indicate the primary formation of succinate from acetate, presumably via the Thunberg reaction. There are several lines of evidence against this.

The work of Gilvarg & Davis (80) already referred to, showed that a mutant of *E. coli* deficient in the citrate condensing enzyme was unable to utilize acetate, though it could form acetyl coenzyme A from acetate, and though it readily utilized succinate. No direct formation of succinate from acetate could therefore occur in this organism.

Swim & Krampitz (227) incubated suspensions of *E. coli* anaerobically in the presence of fumarate and 2-¹³C acetate. Under these conditions, succinate accumulates by the overall reaction (133)

this being the sum of the component processes:

fumarate
$$+$$
 H₂O \rightarrow malate \rightarrow oxalacetate $+$ 2H oxalacetate $+$ acetate \rightarrow citrate \rightarrow citrate \rightarrow isocitrate isocitrate \rightarrow isocitrate \rightarrow a-ketoglutarate $+$ CO₂ $+$ 2H α -ketoglutarate $+$ H₂O \rightarrow succinate $+$ CO₂ $+$ 2H \rightarrow 3 fumarate $+$ 6H \rightarrow 3 succinate

If these reactions are the sole route from acetate to succinate, isotope from the added 2-13C acetate should be found in only one of the two methylene groups of succinate, and the evolved carbon dioxide should be devoid of ¹³C. Analysis of the reaction products showed that this was the case: no isotope was incorporated into the evolved carbon dioxide and ethylene, obtained from the decarboxylation of the formed succinate, was a mixture of molecular species of masses 28 and 29. Had any succinate been formed via the Thunberg condensation this succinate would have been labelled in both

methylene groups and would have produced ethylene of configuration $^{13}\text{CH}_2$: $^{13}\text{CH}_2$ and mass 30. The fact that no ethylene of this mass was detected again shows that the Thunberg condensation does not occur in *E. coli*; these results provide further proof that the reactions of the TCA cycle were the only route from acetate to succinate under these conditions.

A similar interpretation can also be placed on the earlier work of Kalnitsky, Wood & Werkman (103), in which *E. coli* extracts were found to catalyze the anaerobic formation of 1-13C succinate and unlabelled acetate and formate from unlabelled pyruvate, carbon dioxide, and 1-13C acetate. In addition to the component reactions described above, the reaction:

pyruvate + H₂O → acetate + formate

occurred under these conditions. (Since, in this decomposition of pyruvate the 2H produced appeared in formate, considerably less succinate would be produced than when acetate plus fumarate were the substrates as was, in fact, observed.) Again, the experimental evidence fully agrees with a formation of succinate from acetate via reactions of the TCA cycle and provides no proof for the occurrence of the Thunberg reaction.

In the light of these interpretations, it is likely that the primary formation of labelled succinate from labelled acetate noted in the experiments of Glasky et al. (81, 82) was a result of the use of thick suspensions of E. coli in which partial anaerobiosis would effect the overall reaction vii. No such preferential incorporation of isotope into succinate was observed when dilute suspensions of E. coli, growing on acetate, were incubated with ¹⁴C acetate for brief periods (130).

Experiments on the incorporation of labelled acetate by acetate-grown *Pseudomonas* showed that, at the earliest times of sampling, isotope was incorporated into malate as well as into citrate (114, 115). The results obtained indicated a second point of entry into the TCA cycle and suggested the occurrence of reactions of some intermediates of the TCA cycle ancillary to the cycle.

Two such reactions had been reported. Campbell, Smith & Eagles (36) noted that extracts of *Pseudomonas aeruginosa* catalyzed the formation of glyoxylate and succinate from *cis*-aconitate or citrate. Further studies, particularly by Olson (175, 176), Saz (197, 198), and Smith & Gunsalus (207, 208, 209) have shown this reaction to be an aldol cleavage of isocitrate, catalyzed by the enzyme variously referred to as isocitriclyase (175, 176), isocitritase (125, 126, 127, 207 to 210), isocitratase (34, 129), or isocitrase (63):

HOOC·CH₂·CH₂(COOH)·CH(OH)COOH isocitric acid

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i.

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of ne

h

⇒ HOOC·CH₂·CH₂·COOH + OHC·COOH viii. succinic acid glyoxylic acid

Since the substrate of cleavage is isocitrate, the term "isocitratase" is preferred by this reviewer. A second reaction was the formation of malate by extracts of *E. coli* incubated with acetyl phosphate, phosphotransacetylase, coenzyme A, and glyoxylate, first demonstrated by Wong & Ajl (260). This reaction, catalyzed by malate synthetase, has been shown to be a condensation of acetyl coenzyme A with glyoxylate to form malate (34, 116, 118, 126, 127, 131, 261):

 $OHC \cdot COOH + CH_3 \cdot CO \cdot S \cdot CoA + H_2O \rightarrow HOOC \cdot CH_2 \cdot CHOH \cdot COOH + CoA \cdot SH \ ix.$

Both reactions viii and ix were readily demonstrated with extracts of acetate-grown *Pseudomonas* (125, 126, 127) by showing the incorporation of isotope from labelled acetate into malate in the presence of ATP, coenzyme A, glutathione, and either glyoxylate or isocitrate, and by showing the net formation of malate and succinate from acetate and isocitrate in this system, via the overall reaction x.

acetyl coenzyme A + isocitrate → malate + succinate

x.

When acetate was omitted, the extracts catalyzed a stoichiometric cleavage of isocitrate to succinate and glyoxylate; in the presence of acetate, succinate and malate were formed (127). In addition, the extracts contained malic dehydrogenase, condensing enzyme, and aconitase. These latter enzymes, acting in conjunction with isocitratase and malate synthetase, form a cyclic system, one turn of which results in the formation of one molecule of succinate from two molecules of acetate. This cycle was termed "the glyoxylate by-pass of the TCA cycle" by Kornberg & Madsen (125) and "the glyoxylate cycle" by Kornberg & Krebs (121). The evidence for the occurrence of this cycle may be summarized as follows:

(a) Microorganisms growing on acetate as the sole carbon source initially incorporated isotope from labelled acetate into both malate and citrate, and the extent of labelling of malate was such that it could not have been derived

from the oxidation of citrate via succinate (114, 115, 118).

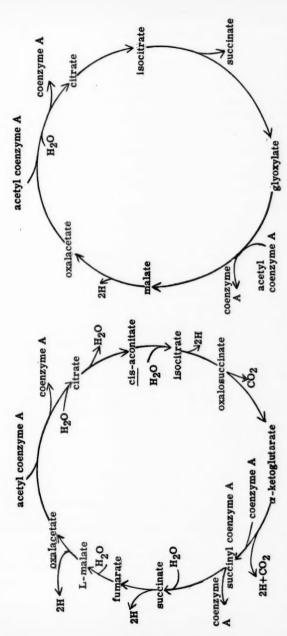
(b) The distribution of isotope within amino acids isolated from the cells was in accordance with the operation of the cycle, and was not in agreement with that of the TCA cycle as the sole metabolic path of acetate (128).

(c) All the enzymes of the glyoxylate cycle have been shown to be present in extracts of acetate-grown cells, and the rates of the individual reactions were quantitatively sufficient to account for the observed rates of growth on

acetate (118, 127, 130).

(d) One of the key enzymes of the cycle, isocitratase (reaction viii), has been shown to be adaptively formed only under conditions necessitating net synthesis of C₄ compounds from acetate (34, 129, 208). When cultures of *Pseudomonas* were transferred from a growth medium containing succinate as the sole carbon source to one containing acetate, *de novo* formation of isocitratase preceded the growth of the organisms (124, 129).

(e) Other postulated pathways, leading from acetate to intermediates of the TCA cycle, which depend on CO₂-fixation reactions (1, 195) or the Thunberg condensation (reaction vi) were not found to occur in experiments with



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Fig. 1. The tricarboxylic acid and glyoxylate cycles. Substances which enter the cycles (coenzyme A, H₂O) after the initial condensation of one molecule of acetylcoenzyme A and one molecule of oxalacetate are written inside the cycles; substances which arise are written outside.

whole cells or extracts thereof. The "direct" oxidation of acetate to glyoxylate (27, 28) has been found to be mediated by isocitratase (29).

Comparison of the TCA and glyoxylate cycles (Fig. 1) shows that the intermediates of the latter (save for glyoxylate) are also those of the former, which provides an explanation for the fact (83, 115) that despite the inability of the TCA cycle to explain the growth of organisms on acetate, only these intermediates and amino acids derived directly therefrom acquire label from isotopic acetate in short-term experiments. The glyoxylate cycle differs from the TCA cycle chiefly in by-passing the oxidative steps from isocitrate to succinate: no CO₂ is evolved, and net formation of a C₄ compound from two molecules of acetate is possible. It is significant also that the overall reaction of the glyoxylate cycle is formally identical with the Thunberg reaction vi; the mechanism is, however, entirely different.

THE METABOLIC ROLE OF THE GLYOXYLATE CYCLE

The glyoxylate cycle accounts for the net synthesis of C₄ dicarboyxlic acids from acetate when this is the sole source of carbon and it therefore provides the oxalacetate required for the continued operation of the TCA cycle. It is also a key step in the synthesis of many cell constituents from acetate. The succinate formed can be utilized for the synthesis of hexoses, via succinic dehydrogenase, fumarase, reactions iii or iv and a reversal of glycolysis [for review, see (137)], by the route:

 $\begin{array}{cccc} (glyoxylate & (succinic de-\\ cycle) & hydrogenase) & (fumarase) & or iv) & glycolysis) \\ 2 & acetate \rightarrow succinate \rightarrow fumarate \rightarrow malate \rightarrow phosphopyruvate \rightarrow hexoses \end{array}$

Hexoses, in turn, can supply pentoses through the pentose phosphate cycle, as well as the carbon skeletons of aromatic amino acids. The intermediates of the TCA cycle, which can be continuously synthesized from acetate via the glyoxylate cycle, can be utilized for provision of the carbon skeletons of many amino acids (see section, Dual Role of the TCA Cycle). The operation of the glyoxylate cycle can also explain the mechanisms of formation of organic compounds, accumulated during growth on acetate by many microorganisms.

The formation of labelled malate from ¹³CO₂ by ethanol-grown *E. coli* was observed by Nishina, Endo & Naskayama in 1941 (163). Foster and his colleagues (72, 74) similarly observed incorporation of label from ethanol into fumarate accumulated by *Rhizopus nigricans* growing aerobically on ethanol. It was noted that 1-¹⁴C ethanol gave rise to fumarate labelled exclusively in the carboxyl groups, but that the specific activity of the incorporated carbon was lower than that of ethanol from which it was derived (74). 2-¹⁴C Ethanol gave rise to fumarate labelled in both the methene and carboxyl carbons, the labelling being in the proportion of 36:7 and the specific activity of each methene group being equal to that of the methyl group of ethanol. The entrance of isotope into the carboxyl groups was not caused by CO₂ fixation, and was ascribed to a reversible decarboxylation of pre-existing oxalacetate or malate in equilibrium with the fumarate (74). Since between 50 to 80 per

cent of the added ethanol could be converted to fumarate, this explanation would demand an enormous reservoir of unlabelled oxalacetate in the mold. A further difficulty was that the fumaric acid formed contained more radioactivity than the sum of two molecules of ethanol when 2-14C ethanol was the substrate, while it contained less when 1-14C ethanol was used. Studies on the exchange of the carboxyl groups of fumarate with labelled CO₂ led Foster & Carson (72) to postulate a net formation of succinate via the Thunberg reaction from two C₂-units derived from ethanol, coupled with exchange of the carboxyl groups thus formed with CO₂. The extent of this exchange appears, however, to be less than would be expected from the observed incorporation of isotope in the carboxyl groups of fumarate.

These difficulties can be resolved if it is assumed that the net formation of fumarate was a result of the metabolism of acetyl coenzyme A, derived from ethanol, via the TCA and glyoxylate cycles. The oxidation of 2-14C ethanol via the TCA cycle would, after more than five turns of the cycle, result in an isotope distribution in fumarate such that the methene carbons are at the same specific activity as the 2-carbon of ethanol, while the carboxyl carbons have half this activity. The glyoxylate cycle as the sole pathway would supply isotope from 2-14C ethanol only into the methene carbons. A combined action of the two pathways would therefore be expected to result in an isotope distribution in fumarate part way between that expected from the sole operation of the two cycles, as actually observed. In support of this belief is the demonstration by Olson (175) of the presence of isocitratase in Rhizopus nigricans. The accumulation of fumarate from ethanol could thus involve the steps:

 $\begin{array}{c} 2 \; ethanol + 2O_2 \rightarrow 2 \; acetate + 2H_2O \\ acetate + oxalacetate \rightarrow citrate \\ citrate \rightarrow isocitrate \\ isocitrate \rightarrow succinate + glyoxylate \\ succinate + \frac{1}{2}O_2 \rightarrow fumarate \\ acetate + glyoxylate \rightarrow malate \\ malate + \frac{1}{2}O_2 \rightarrow oxalacetate \\ \end{array}$

Sum: 2 ethanol + 3O₂ → fumarate

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The validity of this explanation for the earlier findings has been accepted and its implications have been discussed by Foster (71). It can be applied also to other conditions in which molds, growing on ethanol or acetate, excrete intermediates of the TCA cycle or substances directly derived therefrom. Thus, citric acid accumulated by acetate-grown Aspergillus niger was calculated by Lewis & Weinhouse (143) to be derived 40 per cent from recycled C_4 acids and 40 per cent from C_2+C_2 condensations. The observed patterns of isotope incorporation are in agreement with those expected from the combined operation of the TCA and glyoxylate cycles; both the occurrence of the latter pathway in whole cells, and of the enzymes thereof in extracts, of Aspergillus niger have been demonstrated (118). In this case, the sequence of reactions could be written:

 $\begin{array}{c} acetate + isocitrate \longrightarrow succinate + malate \\ malate + \frac{1}{2}O_2 \longrightarrow oxalacetate \\ succinate + O_2 \longrightarrow oxalacetate \\ 2 \ acetate + 2oxalacetate \longrightarrow 2 \ citrate \\ citrate \longrightarrow isocitrate \\ \end{array}$

Sum: 3 acetate + 1½O2 → citrate

Similarly, the association of glyoxylic acid with the metabolism of C₂ compounds in other molds, noted particularly in the work of Walker and Challenger et al. (42, 245), Bernhauer (23), Nord & Vitucci (164), Smith (211), and Weinhouse (247), and the demonstration of one of the key enzymes of the glyoxylate cycle, isocitratase, in the molds Penicillium chrysogenum (175), Rhizopus nigricans (175), and Aspergillus niger (118, 175), point to the significance of this cycle as one of the pathways of C₂ metabolism in these organisms.

The growth of microorganisms on substrates, the degradation of which yields acetate or acetyl coenzyme A, poses similar problems to those discussed for the growth of organisms on acetate; again the carbon skeletons of all cellular constituents must be derived from C2-units. Callely, Dagley & Hodgson (33, 34) found that extracts of a Vibrio grown on octanoate as the sole carbon source oxidized even-numbered fatty acids to acetate, and that acetate oxidation was promoted by the addition of fumarate or glyoxylate. The rate of oxygen uptake observed with a mixture of acetyl phosphate and glyoxylate was greater than the sum of the rates of oxidation of the compounds separately. Moreover, the extracts contained high levels of isocitratase and malate synthetase activity. These findings provide good evidence that the glyoxylate cycle is a stage in the conversion of fat to other cell materials [cf. (117)]; for example, the conversion of fat to carbohydrate may be written:

Similar considerations apply to the route of synthesis of cell constituents by organisms growing on 2:3-butanediol, which was shown by Juni & Heym (101) to be converted to acetate via a "2:3-butanediol cycle." The experiments of Dagley (51) with a soil organism grown on this substrate as sole carbon source show that the isocitratase content under these growth conditions was as high as when the acetate was the substrate of growth, and that it is therefore likely that the biosynthesis of cellular material from 2:3-butanediol is closely analogous to biosynthesis from acetate.

Further evidence for the role of the glyoxylate cycle in acetate metabolism comes from a study of the labelling patterns of glucose synthesized from 1-C¹⁴ acetate by *E. coli* (17). As expected from the operation of the TCA and glyoxylate cycles and, in particular, the overall reaction xi, isotope was found to be significantly incorporated only into carbons 3 and 4. These findings also provide additional evidence against the formation of cell materials by combination of acetate with a C₁-unit derived from acetate. Similar conclusions may also be drawn from the labelling of the glucose units of

starch, synthesized from labelled acetate by the flagellate protozoan *Polytomella coeca* reported by Barker & Bourne (18). Isotope from 2-14C acetate was incorporated to an equal extent into carbons 1, 2, 5, and 6 and, to slightly less than half this extent, into carbons 3 and 4, while isotope from 1-14C acetate appeared exclusively in carbons 3 and 4. Again, the probable route of starch synthesis from acetate would be via the TCA and glyoxylate cycles, and the labelling patterns observed are those expected from the occurrence of reaction xi. In this context, it is of particular interest that the glyoxylate cycle has been shown to operate in the phytoflagellate *Polytoma wella* (181).

In studies of acetate incorporation into ribose by *Alcaligenes faecalis*, Brenneman (31) found cell extracts to contain malate synthetase and to incorporate added glyoxylate into carbons 1 and 2 of the pentose. This suggests that the pentose arose via the steps

 $acetate + glyoxylate \rightarrow malate \rightarrow phosphopyruvate \rightarrow hexose \rightarrow pentose$

The isocitratase content of the cells under these conditions was not reported.

These results, and the widespread distribution of isocitratase and malate synthetase (Table II) indicate the biological importance of the glyoxylate cycle as a means of supplying from acetate the carbon skeletons of cell constituents synthesized during the growth of microorganisms. Studies of the effect of growth substrates on the levels of these enzymes found within microorganisms (33, 34, 124, 129, 208, 261) indicate that the cycle is probably

TABLE II

SOME ORGANISMS IN WHICH THE KEY ENZYMES OF THE GLYOXYLATE CYCLE
HAVE BEEN SHOWN TO OCCUR

Organism	Reference	Organism	Reference
ISOCITRATASE		Serratia marcescens	(208)
Acetobacter aceti	(208)	Vibrio	(33, 34)
Aspergillus niger	(118, 175)	MALATE SYNTHETASE	
Azotobacter vinelandii	(208)	Aerobacter aerogenes	(261)
Baker's yeast	(175, 176)	Alcaligenes faecalis	(31)
Escherichia coli	(208, 259)	Aspergillus niger	(118)
Micrococcus denitrificans	(119)	Bakers' yeast	(116)
Mycobacterium butyricum	(160)	Chromatium	(205)
Penicillium chrysogenum	(175)	Cornyebacterium creatinovoran	s (261)
Polytoma uvella	(181)	Escherichia coli	(260)
Pseudomonas aeruginosa	(36, 208)	Micrococcus denitrificans	(119)
Pseudomonas fluorescens	(125, 127,	Polytoma uvella	(181)
	198, 208)	Pseudomonas fluorescens	(125, 127)
Pseudomonas ovalis	(129)	Pseudomonas ovalis	(124)
Pseudomonas putrifaciens	(208)	Rhodopseudomonas spheroides	(123)
Rhizopus nigricans	(175)	Saccharomyces drosophilarum	(131)
Saccharomyces drosophilarum	(131)	Vibrio	(33, 34)

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n A not a universal route from all C2 compounds to cell material, but that it operates only when the carbon source is acetate or some material (like fatty acids or 2:3-butanediol) the degradation of which yields acetate as its chief product. Smith & Gunsalus (208) noted that growth on glucose or anaerobiosis substantially reduced the isocitratase activity of a number of bacterial species. Callely et al. (34) and Dagley (51) similarly found that the isocitratase activity of their organisms when grown on acetate, fatty acids, or 2:3butanediol was greater than when these organisms were grown on succinate, benzoate, or p-hydroxybenzoate. Like results were obtained with Saccharomyces drosophilarum (131) and Pseudomonas ovalis (124, 129); cells grown on glucose, citrate, succinate, lactate, fumarate, malate, glycollate, glycine, alanine, aspartate, or glutamate as the sole source of carbon were found to contain less than 10 per cent of the isocitratase activity of cells grown on acetate. Although acetate-grown cells continued to synthesize the enzyme when growing on fresh acetate growth media, the addition of succinate to such media prevented the further formation of isocitratase [cf. (152, 178, 179, 243, 264)]. Amounts of the other key enzymes of the glyoxylate cycle, malate synthetase, did not vary with growth substrates as much as did the isocitratase (124, 131) and the enzyme has been found in all microorganisms examined under all conditions of aerobic growth. Experiments on Saccharomyces drosophilarum (131) showed that while change from growth on acetate to growth on succinate or citrate was accompanied by more than a twentysevenfold reduction in the isocitratase content of cell extracts, malate synthetase activity suffered only an eightfold reduction. The considerable activity of malate synthetase in microorganisms not grown on acetate may indicate the existence of metabolic sources of glyoxylate other than the cleavage of isocitrate (37, 38).

THE METABOLISM OF OTHER C2 COMPOUNDS AND OF FORMATE

Metabolism of glycine, glycollate, and oxalate.—The observation that isocitratase is present in high concentration only in cells grown on acetate or on direct precursors of acetate makes it unlikely that the glyoxylate cycle operates as a biosynthetic pathway during the growth of organisms on C₂ compounds other than acetate. The presence of malate synthetase in such organisms suggests, however, that this enzyme may be involved in biosynthetic reactions provided that the metabolism of the C₂ substrate involves the formation of glyoxylate.

The formation of glyoxylate from the oxidation of glycine was already noted by Janke & Tayenthal (94) in 1937. Campbell (39) confirmed this observation, and postulated the oxidation of glycine by a pseudomonad to proceed by the reaction sequence:

$$\begin{array}{l} \text{glycine} + \frac{1}{2} O_2 \rightarrow \text{glyoxylate} + \text{NH}_2 \\ \text{glyoxylate} + \frac{1}{2} O_2 \rightarrow \text{formate} + \text{CO}_2 \\ \text{formate} + \frac{1}{2} O_2 \rightarrow \text{CO}_2 + \text{H}_2 O \end{array}$$

Sum: glycine $+ 1\frac{1}{2}O_2 \rightarrow 2CO_2 + NH_3 + H_2O$

In the second step of this sequence, the α -carbon of glycine was postulated to yield carbon dioxide and the carboxyl carbon to give rise to formate. In the presence of hypophosphite, known to be an inhibitor of formic dehydrogenase (228), formate accumulated and the labelling patterns observed when labelled glycine was used, supported this reaction sequence. Bachrach (16) observed no inhibitory action of hypophosphite, and further suggested that the first reaction of the sequence was not a direct oxidation, but a transamination process, since glycine was metabolized only slowly by dried cells and not at all by cell-free preparations unless ATP, pyridoxal, and α -keto-glutarate were added.

The formation of glyoxylate from glycollate was suggested by the work of Wright (263) with a mutant of *Neurospora* requiring glycine or serine for growth. It was observed that this nutritional requirement could also be met by glycollate or glyoxylate, and Wright concluded (263) that glycollate was oxidized to glyoxylate, which was then aminated to glycine. Extracts of *Pseudomonas* and *E. coli* grown on glycollate as the sole carbon source also readily form glyoxylate (120) and oxidize glycollate, glyoxylate, and formate.

Preliminary results obtained with a pseudomonad grown on oxalate (Quayle, personal communication) indicate that glyoxylate may also arise

from oxalate.

The possible involvement of glyoxylate in oxalate metabolism is of particular interest in view of earlier suggestions (24, 97) that growth on oxalate may proceed by a primary oxidation of oxalate to carbon dioxide and a subsequent fixation of this compound by mechanisms similar to those operating in photosynthetic and autotrophic organisms [see (242)]. The occurrence of such mechanisms would demand the presence in oxalate-grown cells of carboxydismutase (190), the enzyme catalyzing the carboxylation of ribulose 1:5-diphosphate. Furthermore, cells growing on oxalate should incorporate large quantities of isotope from added labelled carbon dioxide, this incorporation (in brief incubations) occurring chiefly into phosphorylated compounds. Studies on Pseudomonas oxalaticus by Quayle & Keech (188, 189) showed that cells grown on oxalate contain negligible quantities of carboxydismutase. The incorporation of labelled carbon dioxide during growth on oxalate was small, and the isotope appeared mainly in malic acid. These findings render it unlikely that carbon dioxide fixation processes play a major role in the metabolism of oxalate. Jakoby, Ohmura & Hayaishi (96) found that extracts of an oxalate-grown bacterium catalyzed an anaerobic decarboxylation of oxalic acid to formic acid, which was dependent on the presence of substrate amounts of ATP and catalytic quantities of acetate, coenzyme A, thiamine pyrophosphate, and magnesium ions. Jakoby & Bhat (95), on the basis of these results, suggested that " . . . it would, then, be formate rather than oxalate which serves the organism as both carbon and energy source." Studies on the metabolism of formate (see following section) render this suggestion also unlikely. Although little is as yet known of the details of the biosynthetic routes from oxalate to cell materials, it appears probable that these routes are similar to those operating in cells grown on glycollate or glycine, and are unlike those occurring in cells growing on C₁ compounds.

The observation that some organisms grown on glycine, glycollate, or oxalate appear to share the property of transforming the growth substrate into glyoxylate suggests that the pathways of biosynthesis from these substrates may also contain reactions common to all of them. This impression is strengthened by the observation that an enzyme catalyzing the anaerobic decarboxylation of glyoxylate, first described by Krakow & Barkulis (139), is found in high activity in extracts of cells grown on these three C₂ compounds but appears to be absent from cells grown on acetate or other carbon sources. These workers suggested that the decarboxylation of glyoxylate noted with extracts of glycollate-grown *E. coli* consisted of the formation of hydroxypyruvate or tartronic semialdehyde from glyoxylate, by transfer of a C₁-unit, accompanied by evolution of CO₂:

2 glyoxylate
→ CO₂ + tartronic semialdehyde

hydroxypyruvate xii.

Addition of unlabelled hydroxypyruvate (61) to cell extracts catalyzing the almost quantitative evolution of labelled carbon dioxide from 1-14C glyoxylate does not lead to incorporation of isotope into hydroxypyruvate, although a small quantity of labelled material, the 2:4-dinitrophenylhydrazone which behaves somewhat similarly to that of hydroxypyruvate, is formed (120). The known instability (43) of tartronic semialdehyde and of its possible precursors, e.g., oxaloglycollic acid, may explain both the nonaccumulation of a labelled keto acid under these conditions and the formation of glycolaldehyde through subsequent decomposition of this compound (51). Tartronic semialdehyde has been implicated in the metabolism of dglucuronate by Aerobacter aerogenes (155). If the presence of enzymes catalyzing the formation and the utilization of glyoxylate in organisms grown on glycine, glycollate, or oxalate is indeed indicative of a shared pathway for biosynthesis of cell materials from the C2 substrates, such a pathway may be visualized as in Figure 2. It should be emphasized that many of the reactions pictured therein have not been demonstrated and that the scheme is therefore a hypothetic one.5 This reaction sequence, which would presumably operate in addition to the TCA cycle, would be consistent with Jayasuriya's observation (98) that citrate, which accumulated in the presence of fluoroacetate (102), incorporated isotope from 2-14C glycollate preferentially to that from 1-14C glycollate.

Experiments by Geller (77) indicate that if the scheme in Figure 2 occurs in some microorganisms, it is not the only pathway of biosynthesis from glycine. By using a coryneform bacterium, isolated from soil, which grew readily on glycine as sole source of both carbon and nitrogen, he found that

⁵ The main reactions outlined in Figure 2 have been demonstrated in *Pseudomonas* grown on glycollate (120), glycine (Callely & Dagley, *Nature*, in press), and oxalate (Quale & Kesch, *Nature*, in press) as sole carbon sources.

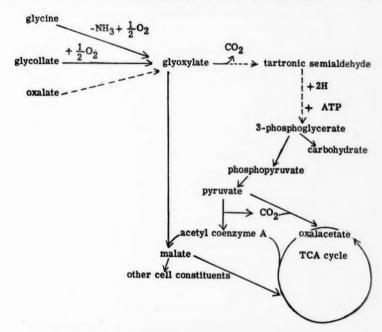


Fig. 2. Hypothetic scheme for the biosynthesis of cell constituents from glycine, glycollate, or oxalate.

extracts prepared therefrom did not catalyze the anaerobic decarboxylation of glyoxylate (reaction xii). Cell-free extracts catalyzed the formation of carbon dioxide, pyruvate, and alanine from glycine in the presence of DPN, TPN, pyridoxal phosphate, and tetrahydrofolic acid. Whole cells incubated with 1:2-14C glycine incorporated isotope initially into serine, alanine, and glutamic acid. These findings suggest that the pathway of biosynthesis from glycine in this organism is similar to that described for *Diplococcus glycino-philus* and *Clostridium acidiurici* by Gunsalus & Sagers (85), and may be visualized as an oxidation of glycine (in the presence of DPN, pyridoxal phosphate, and tetrahydrofolic acid) to carbon dioxide and hydroxymethyl-tetrahydrofolic acid. A C₁-unit derived from hydroxymethyl tetrahydrofolic acid could then be postulated to react with glycine to yield serine; subsequent dehydration and transamination reactions, known to occur in the bacterial extracts, would result in the formation of pyruvate and alanine.

Metabolism of formate.—Recent studies of the routes whereby cells synthesize cell material from formate as sole source of carbon have shown that these are similar to the mechanisms operating in photosynthetic and auto-

trophic organisms. Quayle & Keech (187) found that *Pseudomonas oxalaticus*, growing on formate, rapidly incorporated isotope from either labelled formate or labelled carbon dioxide and that, in brief incubations, 80 to 90 per cent of the incorporated isotope was present in 3-phosphoglyceric acid. The passage through the culture, growing on labelled formate, of unlabelled carbon dioxide reduced the incorporation of isotope to less than 6 per cent of that noted in the absence of unlabelled carbon dioxide, although there was no inhibition of growth as a result of addition of CO₂. Furthermore, extracts of cells grown on formate contained high levels of activity of three enzymes characteristic of the Calvin CO₂-fixation cycle (242); phosphoriboseisomerase phosphoribulokinase, and carboxydismutase. These results contrast sharply with those obtained with the same organism grown on oxalate (see preceding section) and support the view that growth on formate is similar to autotrophic growth, but unlike growth on C₂ compounds.

It is clear that our knowledge of both the catabolic and anabolic reactions of C₁ and C₂ compounds is far from complete. The inhibition of oxidations observed after addition of fluoroacetate (102) and the demonstration that *Pseudomonas* grown on C₂ compounds readily oxidizes all intermediates of the TCA cycle (98, 115, 120), make it probable that the catabolism of these materials proceeds, at least in part, via the TCA cycle. Problems such as the route whereby C₁ compounds other than carbon dioxide or formate (e.g., methane, methanol, or methylamine), or C₂ compounds other than acetate, enter this cycle are still unsolved. The role of the TCA cycle and its ancillary reactions in the photometabolism of microorganisms [see (50, 68, 78)] also remains to be elucidated.

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BIOLOGY OF TETRAHYMENA1,2

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The genus Tetrahymena includes several species of holotrichous ciliates, the most celebrated of which is T. pyriformis. Since very excellent comparative studies of the different species in this genus have been made by Corliss (9, 10), the present report will include only recent work that has been done on T. pyriformis. Corliss (11) has also listed the various areas of research where members of this genus have been used, including a rather complete bibliography of the work completed up to 1954. Other reviews have appeared on protozoan biochemistry, nutrition, and metabolism, large sections of which have been devoted to this ciliate (52, 54, 58, 96). The reader, therefore, is referred to these reviews for the earlier work and, owing to space limitations, only selected recent investigations will be referred to in this report.

THE ORGANISM

The holotrichous ciliate, *T. pyriformis*, was first described by Ehrenberg in 1830 (20) under the name of *Leucophrys pyriformis*. During the ensuing years it acquired many synonyms and this state of confusion existed until the last decade during which its present name has become generally accepted [see Corliss (11)].

In natural habitats T. pyriformis is a bacteria-feeder, but under laboratory conditions it can be grown axenically in ordinary bacteriological media or in chemically defined media. With one exception (50), cysts have never been reported from laboratory cultures, in spite of numerous attempts to induce them by the usual techniques [(24a) unpublished data]. The ciliate measures approximately $50\times30~\mu$ although there is considerable variation in these dimensions among the different varieties and even in the same strain under different conditions. It is typically pyriform in shape with 17 to 21 rows of cilia. The mouth possesses four membranous structures from which it derives its generic name, one undulating membrane being located on the right and three membranelles on the left dorso-lateral wall of the buccal cavity. The entire buccal apparatus is typical for the suborder Tetrahymenina (9, 10, 12).

Its nuclear components consist of an irregularly spherical macronucleus about 10 μ in diameter lying near the center of the cell, and usually a spherical micronucleus (1 to 2 μ) partially embedded in the macronucleus. It is generally agreed that the macronucleus is essential for life of the cell and is

¹ The survey of the literature pertaining to this review was concluded in January, 1959.

 $^{^2}$ The following abbreviations will be used: DNA (deoxyribonucleic acid); DPNH (diphosphopyridine nucleotide, reduced); LD₅₀ (median lethal dose); RNA (ribonucleic acid).

concerned with metabolic processes, whereas the micronucleus is functional during the sexual cycle and is responsible for the interchange of genetic material between diverse cell lines. The existence of many amicronucleate cells in nature indicates the nonessentiality of this structure. A life cycle, involving both sexual and asexual phases, is present.

Only slight morphological differences have been observed among the several thousand strains examined in our laboratories. Likewise, the nutritional requirements, with the rare exceptions discussed elsewhere in this report, are remarkably constant. However, the sexually active strains usually fall into distinct groups (varieties) which are genetically isolated from one another. Each of these can be distinguished serologically using immobilization as the criterion for separation. Loefer et al. (57) were able to separate 31 well-known strains (some with and some without micronuclei) into 14 serological "groups." Strains WH6, WH14, and WH52, mating types I, II, III of variety 1, fell into a single group. We have prepared antisera for representative mating types of most known varieties and find that they can readily be distinguished from each other (24a). The question of whether these differences are sufficiently great to warrant species status has been considered elsewhere (25).

DISTRIBUTION

T. pyriformis was probably seen by most early microscopists who explored the protozoa from fresh-water habitats. In recent times it has been found in nearly all regions where intensive collections have been made (34, 36, 43). The so-called "classical" amicronucleate strains were all found in the United States with the exception of four, three of which were found in France and one in England (9, 10). Sexually active strains have been collected from the United States, Canada, Mexico, Panama, and Colombia (36). Recent collections from several European countries also contain this organism (24a). It is found in moving and standing water, small streams, ponds, and large bodies of water. It is known to exist even as a facultative parasite (101). It has been taken from natural habitats at temperatures ranging from 4° to 30°C., and under laboratory conditions some strains thrive abundantly at 37°C. (13). In elevations it has been found from sea level to 10,000 feet. There seems to be almost no fresh-water habitat where this ciliate refuses to live.

For many years T. pyriformis was thought to possess no sexual types because all of the strains which had become popular tools in research were amicronucleate, and consequently had no genetic mechanism for fruitful cross-breeding since cells without micronuclei do not normally conjugate (25, 37). Sexually active strains have been found in natural populations and at present over 40 mating types, distributed among 9 varieties, have been found (34, 36, 43). Analysis of the mating type inheritance has been reported for varieties 1 (71, 72, 74), 2 (49), 8 (76), and 9 (31). Work is well under way with variety 3 by Byrd (24a), and with 7 by Outka (24a). Since variety 5

yields no viable offspring (82) its breeding system is unknown. No information is yet available for varieties 4 and 6.

The mating type system for variety 1 was first worked out by Nanney and his associates (71, 72, 74). It shows the group A pattern of mating type determination and inheritance. These workers have found, in addition, that a series of alleles at a single locus determines not only different spectra of mating type potentialities but also the frequencies with which these are expressed. Hurst (49) has demonstrated the system in variety 2 to resemble that of *Paramecium bursaria*. Orias has found that the mating types in variety 8 are under direct genetic control (76). Our own observations on variety 9 support a mating type system analogous to Group B of *Paramecium*

aurelia with multiple mating types.

In his excellent review of the breeding systems of ciliates, Sonneborn (100) has included an analysis of the system in *T. pyriformis* based on the evidence available at the time the review was written. The data that have accumulated since seem to support his contention that this ciliate possesses a life cycle consisting of a period of immaturity following conjugation, then a period of sexual maturity during which fruitful conjugation occurs, and finally a period of senility followed by death of the clone. Signs of senility are the appearance of amicronucleate cells or the tendency to mate within the clone (self), or both. Among our thousands of clones obtained from natural habitats we have found from 30 to 50 per cent without micronuclei. Selfers are also found in small numbers from natural collections and often appear among the progeny of certain crosses. Moreover, each year a number of our stocks die under conditions identical for those which thrive. These deaths may well be caused by aging although we have no direct evidence that this is so.

The origin of selfing may be age-induced as suggested by Sonneborn (100). This is borne out by the observations of Wells (105) in variety 6 in which she found that all three of the mating types, once nonselfing strains, mated with themselves. On the other hand, selfers appear in very young clones following conjugation and these very often stabilize into "pure" mating types. This may be accomplished by the segregation of stable and differentiated macronuclear subunits during vegetative division as suggested by Allen & Nanney (5). Their data are employed by Schensted (88) to derive a mathematical model which was based on 45 as the number of diploid subnuclei immediately after cell division. This number obviously does not remain constant throughout the life cycle as indicated from DNA studies reported elsewhere in this paper.

The stability of the gene complex of *T. pyriformis* is revealed when crosses of strains from such distantly located habitats as Panama and Michigan yield viable offspring; few to be sure (1 per cent), yet perfectly normal in every respect. It has not been possible to establish beyond doubt evidence that strains from distantly located areas yield a smaller number of progeny than those from closely located regions. A complicating factor in such studies is

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that one never can be certain of the age of the clone isolated from nature. If it had not conjugated for a long time and hence was to be considered senile, crossing it with any other strain, regardless of location, would result in low viability. This question might be resolved by breeding F_1 clones derived from parents obtained from closely and distantly located habitats.

Signs of degeneration following close inbreeding have been noted in all of our work. Nanney (69) has analyzed this problem in variety 1. He has demonstrated that such anomalies as death at conjugation and failure to complete meiosis rise in frequency with the first few generations, after which survival improves until, by the sixth generation, no lethality is noted. The anomalies which appear in the F_1 generation resulting from crossing naturally occurring strains may arise from chromosome aberrations or from the accumulation of defective genes which, when homozygous, are lethal.

THE MATING PROCESS

The factors influencing the mating process are numerous (37). Starvation is the most significant essential for initiating mating. Washing several times in distilled water accomplishes the most drastic starvation and is also the most effective method of inducing mating. Other factors such as temperature, light, and age of the culture have little effect on the process. Anoxia and agitation prevent mating. Whether or not certain nutrilites of the defined medium might be influential in preventing the mating process is a possibility which Hynes has examined (24a). Any one of the single amino acids, when added in concentrations of 0.1 M to distilled water in which the organisms were suspended, did not prevent conjugation. However, when all of the amino acids were added in the same concentration as in the defined medium, no conjugation occurred. These results suggest that the amino acid effect is not specific. This has been confirmed by Ducoff (17). A mixture of the seven vitamins essential for the growth of T. pyriformis in distilled water reduces conjugation by 75 per cent, while the individual vitamins have no effect. It seems, then, that there is no specific effect attributable to any single amino acid or vitamin. Ducoff (17) reports that acetate, glucose, and the purine-pyrimidine components, alone or in combination, are ineffective in reducing conjugation. We have confirmed these observations (24a).

The mating process itself seems to have no effect on metabolism as indicated by respiratory studies (24a). Strains WH6 and WH14 (variety 1, mating types I & II) were placed in separate compartments of Warburg flasks (one in the sidearm and the other in the flask—then reversing their positions), and oxygen consumption readings were taken for 6 hr. while the cells were separated. The organisms in the test flasks were then mixed and readings taken for the next 24 hr. during which approximately 80 per cent of the cells mated. The controls were not mixed. Slight differences between the control and the test flasks did occur but were probably insignificant, indicating little or no change in metabolism during the mating process. If

subtle changes in metabolism did occur the instrument was not sufficiently accurate to detect them.

On numerous occasions we have attempted to determine the existence of sex hormones which might influence the mating process (24a). Opposite mating types were placed in each arm of a U-tube and separated by a sintered glass filter with size openings small enough to prevent the cells from passing through. The tube was gently rocked in order to insure mixing of compounds in both arms and between them. When such cells were washed and mixed, no shortening of the refractory period (time interval between mixing and beginning of mating) occurred. Extracts of the medium in which the cells were growing, employing a variety of solvents, failed to affect the mating process in any way. If sex hormones do exist we have found no way of detecting them.

Whether or not cytoplasmic exchange occurs between the mates during conjugation is a question which has been partially answered by both morphological and biochemical evidence. Thin sections taken through the area of contact between the mates and examined under the electron microscope demonstrated the presence of well-defined tubules which persist throughout the mating process (40). The diameter of the pores approximates that of the ciliary openings (0.2μ) . It may be assumed that the original pores in the pellicle through which the cilia pass have become modified into pores connecting the two cells. The tubules exist in sufficient numbers (200 to 300) and size and may permit the exchange of cytoplasmic particulates of dimensions up to 0.2 \mu. The entire infraciliature and buccal apparatus dedifferentiate during conjugation and redifferentiate shortly before the cells separate. Autoradiographic studies by Schooley (95) of conjugating WH6 and WH14 have demonstrated that, when one mate was labeled with C¹⁴ aspartic acid, 27 per cent of the pairs showed evenly distributed labeling in both conjugants by the time the conjugants reached the postzygotic division stages (24 hr.). Therefore, it is possible that whatever influence the cytoplasm may have on the nucleus and other parts of the cell, it may be pooled during conjugation, affecting both cells more or less simultaneously.

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In spite of the fact that the classical strains have been popular tools for research for 30 years, almost nothing was done about their cytology until recently, probably because amicronucleate cells, without the processes accompanying conjugation, offer little of interest to cytologists. Maupas (62), however, had described the cytological details relating to conjugation in selfing Leucophrys patula, which is now known (9) to have been T. patula, a species closely related to T. pyriformis. With the rediscovery of selfing strains (39, 48) and the discovery of mating types (34), studies in nuclear behavior during these events became a topic of interest.

The stages of conjugation observed by Maupas were quickly confirmed

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by Nanney (70) and by the reviewer (37). A vivid account of chromosomal behavior was reported by Ray (80). Whereas the events in conjugation are similar to those in other ciliates, the small number and large size of the chromosomes provides a clear picture of meiosis. It seems worthwhile at this point to include a brief account of these events.

Once the cells are well attached in the oral regions, the micronuclei enlarge, forming a sac with unfolded chromosomes which are initially single but later paired. The chromosomes then form a long double thread, lying end-to-end, which extends the length of the cell or even beyond, coiling around the macronucleus. This is the crescent stage. As the crescent shortens, five discrete pairs of chromosomes are clearly visible, three similar in size, one larger, and one smaller, all with median or submedian centromeres. Duplication occurs, forming tetrads which are then separated into dyads at the first meiotic division. The second division follows a brief interval, resulting in four haploid nuclei, only one of which remains functional, the other three disintegrating. The functional nucleus undergoes a third prezygotic division, producing stationary and migratory gametic nuclei. Very shortly the latter from each conjugant moves across the membrane between them and fuses with the stationary nucleus of the reciprocal mate, thus restoring the diploid condition to both animals. During the first postzygotic division the expected 10 chromosomes are clearly visible. The second mitotic division results in four nuclei, two of which lie in the anterior and two in the posterior end of the cell, the former differentiating into macronuclei, and the latter into micronuclei, one of which is soon resorbed. The cytoplasm plays the principal role in determining the type of nucleus that will form in any given locality as demonstrated by Nanney (70). Individual chromosomes are difficult to discern from this point until meiosis is initiated again. A single cell division gives rise to ciliates with one macronucleus and one micronucleus. Vegetation divisions ensue until the next conjugation cycle is initiated.

The chromosome number is the same, haploid five, in all varieties studied so far (31, 37, 81, 83); in addition, the configuration of the individual chromosomes is also similar. Nuclear aberrations associated with conjugation have been reported for variety 5 which may account for the lethality of all cells undergoing the process (83).

Nothing is presently known about the chromosomal events associated with vegetative mitosis of the micronucleus. The macronucleus, however, divides apparently without forming chromosomes that can be seen under the light microscope. The basic structure of the macronucleus of *T. pyriformis*, like that of other ciliates which have been investigated, seems to remain a mystery. Feulgen preparations show many small DNA-positive bodies which might well be "subnuclei" (99), perhaps each with a diploid set of chromosomes. Both light and electron microscope studies in our laboratories, taken throughout both the vegetative and sexual cycles, give no clues as to the nature of these bodies. By following the differentiation of the macronu-

clear anlagen with electron micrographs from the earliest stages to maturity, no chromosomal division figures have been observed, although there is a steady increase in the number of DNA-positive bodies. Associated with this increase in number is a steady increment in the amount of DNA.

Walker & Mitchison (104) have measured the rate of DNA synthesis in strain W and have shown a gradual increase from one generation to the next. McDonald (66) has also measured the synthesis of DNA in another amicronucleate strain (strain H) and has demonstrated that duplication occurs during an intermediate part of interphase, starting some time after the end of a cell division and reaching the maximum amount long before the initiation of the next division. The fact that haploid clones possess the same amount of DNA as do diploids (32) and amicronucleates, supports the idea that there is a minimal level of DNA that must be reached before the cell can divide. However, the upper limits of the quantity of DNA seem to be highly variable. McDonald (66) found that in old mass cultures (1 to 2 weeks) the cells contained approximately double the amount of DNA present in cells in log growth. When such stationary phase cells are placed in fresh media they promptly divide, then go into lag phase before log growth is initiated (79). She also found that the amounts of DNA allotted to sister nuclei at division are very similar although occasional cells showed marked inequality in this respect. Even in these, death did not follow owing, perhaps, to some mechanism that controls the synthesis of sufficient DNA. Apparently the accumulated DNA is sufficient to provide adequate genetic material for one division without synthesis of new DNA. Further evidence in support of this idea is found in the work of Zeuthen & Scherbaum (108) who worked with another amicronucleate strain (GL). They found that cells, at the end of heat treatment which induced synchrony in the next few divisions (94), contained four times as much DNA as normal log phase cells. In the subsequent two divisions the DNA returned to normal. Working with another amicronucleate strain (W), Ducoff has confirmed the high levels of DNA present in temperature-treated cells (18). Recently, however, Scherbaum (91), employing chromatography and spectrophotometry, has found that the DNA is only doubled in 6.5 hr. of heat treatment. This observation was confirmed by Iverson & Giese (51) working with strain W. They also found that the amount remained essentially unchanged during the first division, reaching normal levels only after the second division. Moreover, by the end of the heat treatment, a cell has more RNA than untreated cells, which gradually diminishes in quantity in subsequent divisions until the normal level is reached. Exposure to ultraviolet radiations revealed that heat-treated cells were more resistant than normal cells, and this was correlated with the increased DNA.

It has been known for a long time that small macronuclear fragments appear in the cytoplasm following vegetative division. These fragments gradually diminished in size and finally disappear, following much the same sequence as degenerating macronuclei during conjugation. Recently, they

have been studied by Walker & Mitchison (104) and by Scherbaum et al. (92). They may function in correcting the "imbalance" of macronuclear material incurred during division as suggested by Walker and Mitchison. The DNA in degenerating macronuclei during conjugation may be used again in the regenerating macronuclear anlagen. A similar function might be attributed to these macronuclear fragments during vegetative division.

Roth & Minick (86) have examined the fine structure of the macronucleus of amicronucleate cells (strain not given) in which they find nucleoli. They describe them as dense bodies of 0.3 to 0.5 μ in diameter, embedded in the nucleoplasm. The nuclear membrane is double-layered. In some cells the nucleoplasm is finely granular and filamentous while in others, it is clear with small dense granules about 50 μ in diameter distributed in the network. In these the nucleoli are localized in groups. We have examined the macronucleus of micronucleate strains (WH6 and WH14 and others) during conjugation and have found the small dense nucleoli which Roth & Minick describe but they are crescentic in shape (24a). Their location is consistently peripheral, lying beneath the membrane with a definite orientation to the membrane.

Since the macronuclei are derived initially from micronuclei during conjugation, one would expect the nucleoproteins to be identical. However, Alfert & Goldstein (2), employing a direct staining method for basic protein present per unit of DNA, found in WH6 and WH14 that a difference existed between the nuclear types, the macronuclei staining with relatively less intensity than the micronuclei. This could mean that they differ metabolically and that the micronucleus plays no part in metabolic activity. If so, the survival and hardiness of amicronucleate strains is understandable. We have determined on numerous occasions the superiority of amicronucleate strains to compete in the same test tube with strains with micronuclei (24a). By inoculating equal numbers of both types into stock peptone medium and checking relative numbers over the growth range, the amicronucleates invariably are found to be greater in number in 96 hr. If subcultures are made, none of the micronucleate ciliates remain after the third transfer.

Alfert & Goldstein (2) also report a difference in the size of the micronuclei in WH6 and WH14 (variety 1, mating types I and II), the former exceeding the latter both in size and DNA content. Upon a request by these authors to check the micronuclear size of the original parental strains, we found no difference. Alfert & Balamuth (1) then made a careful cytological study of the same strains and concluded that the larger size and DNA content of WH6 was a case of differential polyteny since there was no increase in number of chromosomes, but only in their individual sizes. This aberration occurred only in the subculture since the original parental strain is not polytenic.

This apparent instability of the genetic material in these aging sexual strains has been observed in our laboratories. Byrd (personal communication) has recently been able to count only four pairs of chromosomes with a

fifth single chromosome during the first meiotic division in variety 1, using the original strains, WH6 and WH14. Ray (81) has observed the spontaneous appearance of tetraploidy in variety 6, mating type II. This may be a sign of aging and it is highly likely that without the intervention of conjugation more and more of these aberrations can be expected.

Mitochondria of *T. pyriformis*, as demonstrated by the electron microscope, contain microvilli and resemble those of other protozoa (98). During vegetative life they are located throughout the cytoplasm. However, we have found them fewer in number and located near the outer cell wall during conjugation (24a). The endoplasmic reticulum is represented by small vesicles and canaliculi irregularly scattered through the cytoplasm. Small bodies which are probably Palade's ribonucleoprotein particles, as well as large lipide bodies, are also present.

Rudzinska (87) has found double cytoplasmic membranes which exist in ovals, circles, or irregular figures arranged concentrically one with the other. They are located near the periphery of the cell or in an indentation in the macronucleus. Nothing seems to be known about their function.

The fine structure of the fibrillar system of T. pyriformis has been described by Metz & Westfall (68). Employing ultrasonic dissections, they found the kinetics of T. pyriformis to be compound structures composed of discrete units, each of which consists of (a) a cilium; (b) a kinetosome; and (c) a tapering kinetodesmal fibril arising from the kinetosome. The latter from each unit unite, with their pointed ends forward, to form the kinetodesma. The system is similar to that found in paramecia and perhaps other ciliates.

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In 1954, Zeuthen & Scherbaum were able to synchronize cell divisions in mass cultures of T. pyriformis by a series of heat shock treatments (94, 108). Eighty-five per cent of the cells were induced to undergo simultaneous division for three generations. This afforded an opportunity to undertake many different kinds of studies in mass cultures. Cell size was determined over the growth and division cycles in both normal and heat-treated cells (89). They started in lag phase as large cells; by eight generations of logarithmic growth the mean cell size was reduced by 50 per cent. However, after 8 hr. of heat treatment the cells were three to four times larger than before the treatment. After 5 hr. of synchronous division the cell size was reduced to normal, although the deviation of cell size within the population differs from that found in a normal culture. Scherbaum & Rasch (93) carried this study further by a careful mathematical analysis of the cell-size distribution of cells in log phase growth. Scherbaum (90) has also shown that the processes of cell growth and those associated with cell division function concurrently but independently of the other, both operating at characteristic rates. It is thought that the temperature cycling which blocks division depends on the reversible heat denaturation of a single enzyme system (47). These authors believe that this enzyme is intimately associated with the movements of cell organelles concerned with morphogenesis and nuclear behavior.

NUTRITION AND METABOLISM

As a result of many years of sustained effort by a number of investigators, the nutritional requirements of *T. pyriformis* are known precisely. This work has been included in several reviews (52, 54, 96), the most recent by Johnson appearing in this publication in 1956. Of the papers, numbering more than 100, which have appeared since this 1956 review, the writer will be able to mention only a few owing to space limitations.

Briefly, the chemically defined medium for *T. pyriformis* includes 11 amino acids, 7 B vitamins, guanine, uracil, and a number of inorganic ions. Fatty acids are not required but lipides, supplied in the form of Tween compounds, are stimulatory. Carbohydrates are unnecessary for growth but they, too, are stimulatory.

The 11 essential amino acids are converted into some 18 amino acids which combine to form peptides and proteins during growth of the organism. Wu & Hogg (106) have shown in their nitrogenous composition studies that nitrogen comprises 7 to 8 per cent of the dry weight of the organism, of which less than one-half is in the form of protein nitrogen while one-third is in the form of peptides or free amino acids. More recently, they have shown that the free and nonprotein α amino nitrogen constitute one-fifth and one-fourth, respectively, of the total nitrogen (107). However, no unusual nitrogen compounds were found.

The lipide content of *T. pyriformis* is high, 15 to 20 per cent of the dry weight (46, 67). Glycogen also is stored in large quantities, 15 to 22 per cent of the dry weight in noncarbohydrate media (60), and varies with the life cycle of the organism. The chemical and enzymatic degradations of the ciliate glycogen showed that it is essentially the same as that of rabbit liver or muscle.

Cell-free preparations of *T. pyriformis* have been profitably used by Mager & Lipmann (59) for studies of the sequence of reactions occurring when amino acids are incorporated into protein. These preparations were particularly favorable for demonstrating the reversibility of transfer of activated amino acid to soluble ribonucleic acid. The amino acid incorporation system of this ciliate is apparently similar to that of higher animals. Pyrimidine synthesis has been investigated by Heinrich, Dewey, & Kidder (44). They have shown that when uracil-2-C¹⁴ is the sole pyrimidine offered to growing ciliates, all of the thymine synthesized by the cells comes from the labeled uracil. Labeled thymine is utilized only slightly as a precursor of DNA thymine.

Kidder and his associates have also investigated the very interesting subject of growth inhibitors, the most recent being the deazapurines (55), one of which, 1-deazaquanine, they synthesized (61). This compound is a growth inhibitor but because of its low solubility, concentrations sufficient to bring about half-maximal inhibition could not be reached. Deazaadenine, however, is a marked inhibitor. The inhibition can be antagonized by adenine. Growth inhibition has also been demonstrated by these authors using the

tryptophan analogue, 7-azatryptophan (56). The analogue appears to interfere with tryptophan utilization. Another important type of growth inhibition is that caused by the amino acids themselves when supplied in unbalanced concentrations. It has recently been shown that all of the amino acids essential for growth of *T. pyriformis* have inhibitory effects except lysine (15). When two or more amino acids are added to the medium simultaneously they act antagonistically in their inhibition. Amino acids in the form of peptides produce small effects on inhibitions caused by other amino acids. Other inhibitors such as colchicine and the steroids, deoxycorticosterone, progesterone, and cortisone, have been shown by Conner & Nakatani (8) to be reversed by stigmasterol. The action of this compound may act in the generation or transport of high-energy phosphate groupings.

It has been known for a long time that *T. pyriformis* produces extracellular proteinases (16, 26, 58). This characteristic has been used to advantage by several workers who employed the ciliate for assaying the biological value of proteins (6, 19, 78, 84). The proteinases produced attack only proteins that have been denatured by heat (103). Viswanatha & Liener (102) have isolated a crystalline extracellular proteinase from strain W which will split denatured hemoglobin as well as other compounds. It differs from pepsin, cathepsin A, and trypsin in that it must be activated by sulfhydryl.

Several intracellular enzymes have been described by a number of investigators whose work was reviewed by Seaman (96). More recently, considerable effort has been made to identify these enzymes in order to elucidate the intermediate metabolic pathways which operate in this ciliate. Many of these studies have been comparative, demonstrating likenesses and differences among bacterial, fungal, protozoan, and mammalian cells.

Employing cell-free homogenates, Eichel (21) has identified spectrophotometrically two purine-metabolizing enzymes, adenosine deaminase and purine nucleoside phosphorylase. Adenase and guanase were absent indicating that *T. pyriformis* cannot directly deaminate the purine bases, adenine and guanine. He also found that the ciliate homogenates deaminate deoxyadenosine, commercial yeast adenylic acid, and 5'-adenylic acid and that the ratio of deamination of adenosine to deoxyadenosine varied with the age of the culture, which implied that two different enzymes are involved.

The controversy over nitrogen excretion in T. pyriformis (16, 53, 75, 97, 106), has been reinvestigated by Dewey et al. (14) who conducted a comparative study of strains E, S, and W in which much of the earlier work was repeated. Three types of studies were conducted: growth, analysis of culture filtrate after growth, and enzymatic analysis of cell-free preparations of ciliates. Tests were made to determine whether any of the following reactions occurred: ornithine—citrulline; citrulline—arginine; arginine—urea+ornithine; urea— CO_2+NH_3 . None occurred to a sufficient extent to account for the known nitrogen metabolism of the organism; hence, according to these authors the Krebs-Henseleit urea cycle does not operate in T. pyriformis, which is in agreement with findings of Wu & Hogg (106).

Work is actively continuing on endogenous respiration and the enzymes involved in this process. McCashland et al. (63, 64, 65) have shown that while growth and respiration of T. pyriformis W is inhibited by relatively high concentrations of KCN, the same processes are actually stimulated by lower concentrations. Moreover, the cells will adapt after continued growth in the presence of KCN so that partial resistance against inhibition is observed. Because individual strain differences in metabolism have been noted by a number of workers these authors made a comparative study of the effect of cyanide on growth and respiration of six amicronucleate strains of T. pyriformis (W, L-I, L-II, HS, ChS, E) and one of Tetrahymena vorax (V₁). This work demonstrates considerable variation among the strains and it is significant that they found no relation between the more active processes in respiration and cyanide sensitivity. For example, strain W grows most rapidly, has high respiratory activity, and is markedly sensitive to KCN, whereas strain E grows very slowly and has low respiratory activity, yet it is also highly sensitive to cyanide. It seems that cyanide sensitivity or resistance involves something more than mere rate of metabolism.

Eichel has undertaken a series of studies of the respiratory enzymes of *T. pyriformis* (22, 23, 24). He has shown that the succinoxidase system differs markedly from the classical scheme of electron transport between succinate and O₂. Using cell-free homogenates of strains S, W, and GL, he was unable to demonstrate cytochrome-*c* activity in the presence of succinate. More recently, he has characterized a DPNH-oxidase system which exhibits DPNH cytochrome-*c* reductase (diaphorase) activity. The components of the electron transport chain differ from those found in the mammalian DPNH-oxidase system. In characterizing the enzyme system in this ciliate he has shown that a phospholipide is associated with the particle-bound enzyme system.

Hogg & Wagner (46) have shown that under aerobic endogenous conditions the glycogen content doubles when the ciliates are fasting. Fermentation of glycogen occurs under anerobiasis. The concurrent loss of either lipide or protein alone accounted for the aerobic glycogen synthesis. However, amino acids failed to stimulate synthesis but fatty acids and triglycerides caused 100 per cent stimulation. Butyrate-1-C¹⁴ was removed almost completely from the medium by mature cells, 10 to 20 per cent of the carbon¹⁴ being incorporated into glycogen, whereas neither glutamate nor ornithine was incorporated significantly (45). Also mature cells incorporated 75 per cent of the carbon¹⁴ of glucose-U-C¹⁴ into glycogen and less than 10 per centradioactivity into CO₂. It is suggested that glycogen deposition may displace the metabolic equilibria toward glyconeogenesis.

Some effort has been made to localize certain enzymes in T. pyriformis employing histochemical techniques. Fennell & Marzke (42) studied cytoplasmic alkaline phosphatase in strain W and found that its distribution and quantity varied with the age of the culture. Later Fennell & Degenhardt (41) demonstrated the possible existence of three phosphomonoesterases

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which hydrolyze glycerophosphate, adenosine-5-phosphate, and adenosine triphosphate. Allen (3) studied the localization of acid phosphatase and aliesterase in both an asexual (strain W) and a sexual (isogenic clones of each of five mating types in variety 1 and a selfer) strain. Characteristic intracellular localization for each enzyme was found but the pattern varied with both temperature and the age of the culture. No differences were noted in the patterns among the sexual clones but these may differ from that of strain W. At conjugation the position of acid phosphatase changes and this may be correlated with the activity of the macronucleus. The activity of both enzymes decreases during the course of conjugation. Employing both cytochemical techniques and starch-gel electrophoresis, she has been able to analyze the esterases more critically (personal communication). She notes differences in inbred strains which, when crossed, yield hybrids with patterns that suggest that the control of esterase activity may be in part genetic.

IRRADIATION

T. pyriformis is convenient for studying the effects of various radiations on cells. It is highly resistant to those that have been tried. Both amicronucleate and micronucleate strains (WH6 and WH14) will tolerate 600,000r in distilled water and have an LD50 of 400,000r. Sexually active strains which receive 200,000 to 600,000r, and then are mated, show gross chromosomal aberrations and usually fail to give rise to viable progeny (38). When those receiving 400,000r are mated with normal nonirradiated cells, some progeny are haploid (32), owing to the destruction of the genetic apparatus in the irradiated cell. By exposing conjugating cells of the serine mutant strains in variety 9 when they are in prophase of the first meiotic division to 400,000r it is possible to obtain progeny which have lost the capacity to synthesize serine. In other words, the gene or genes have been altered sufficiently so that they no longer direct the synthesis of serine. The progeny are, therefore, phenotypically like the naturally occurring serine requirers (33).

The ciliate has also been a convenient test organism for determining radiation damage to foods (27). By exposing the 11 amino acids and 7 B vitamins essential for normal growth of T. pyriformis to γ -radiation from a cobalt⁶⁰ source it was found that thiamine, riboflavin, pantothenic acid, pyridoxine, folic acid, and thioctic acid were destroyed by less than 1×10^6 rep, while 2×10^6 rep was required to inactivate nicotinic acid. Serine and methionine were damaged at 1×10^7 and 2.3×10^7 rep, respectively. All other amino acids were unaffected at the latter level which was the highest employed.

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Roth & Eichel (85) have shown that x-ray doses of 300,000 to 600,000r of cell homogenates resulted in appreciable destruction of the succinic, glutamic and malic dehydrogenase systems, catalase, and DNase. Catalase activity recovered when the cell homogenates were allowed to stand at 0°C. However, whole cells are relatively unaffected by these dosages of x-rays,

although respiration is decreased. Mortality resulting from x-radiation must result from injuries elsewhere in the cell.

GENETICS

The genetics of the ciliated protozoa has reached its greatest height with the work of Sonneborn and his associates [reviewed by Beale (7)] working with *Paramecium aurelia*. Only a beginning has been made with *T. pyriformis*, yet even this limited information is contributing significantly to our knowledge of ciliate genetics.

The first contribution in this field, the inheritance of mating types, was made by Nanney and his associates (49, 71 to 74, 76) and referred to earlier in this paper. These valuable contributions have laid a firm foundation for all future work, because without this knowledge it is extremely difficult, if not impossible, to conduct meaningful analyses of other traits which one might wish to explore.

Morphological mutants are rare in *T. pyriformis*. It is true that one often finds highly abnormal animals, multinucleate, weird shapes, double mouths, and other abnormalities, but when such cells are isolated and clones established from them they usually revert to the normal type, if they survive. However, Allen (4) reports a multinucleate and bizarre-shaped clone isolated by Nanney which seems to be a true morphological mutant. This trait disappears at conjugation. The nature of the defect responsible for this mutant is, as yet, unknown.

Orias (77) has analyzed two lethal conditions in strain IL-1 in variety 1 which are controlled by two unlinked recessive genes, t (phenotype-"tiny") and t (phenotype-"fat"). Both are lethal when homozygous. The epistatic nature of the "tiny" over the "fat" genes is shown by the 9:3:4 phenotypic F_2 ratio, with the "tiny" in excess.

It would seem that *T. pyriformis*, with its known nutritional requirements, the available information on its intermediate metabolism, and now an understanding of its breeding system, would certainly be an ideal animal cell for biochemical genetic studies. This may prove to be so but the numerous hardships encountered in overcoming progeny lethality, mating behavior, and the strict asepsis which is essential in all experiments, introduces difficulties that are burdensome and, at times, frustrating. In spite of these obstacles it has been possible to gain some information about the inheritance patterns of the genes controlling serine and pyridoxine synthesis.

Among 2500 clones taken from many parts of the United States and Canada (43), as well as from Mexico, Panama, and Colombia (36) and screened in our laboratory by the omission technique for the 11 essential amino acids and 7 B vitamins, six clones were found that grew without serine (35) and 41 that survived without pyridoxine (30). Successive weekly transfers for many months in deficient media was considered adequate proof of the synthetic capacity of these mutants. Only one mating type was found among the serine mutants, whereas opposite mating types were available

for study among the pyridoxine mutants. By selection for breeding behavior and other favorable characteristics, it was possible to design breeding experiments.

The question as to whether the factors controlling pyridoxine synthesis are nuclear or cytoplasmic was resolved by crossing the nonrequiring mutant with a requiring wild-type haploid which had previously been produced with x-irradiations (32). The haploid is a genetic blank since it is unable to undergo fruitful meiosis. By mating a pyridoxine nonrequiring diploid with a requiring haploid, it was shown that the clones established from both exconjugants grew without pyridoxine, demonstrating that the gene or genes controlling pyridoxine synthesis were carried in the migratory gametic nucleus.

By making appropriate crosses of serine and pyridoxine requiring and nonrequiring clones, we have been able to establish the genotypes of the clones found in natural habitats (28, 29). The results indicate that the wild-type requirers are heterozygotes (+/s or +/p), whereas the nonrequiring mutants are homozygous recessives (s/s or p/p). Inbred clones were established in which three genotypes for both mutants are known (+/+, +/s, +/p, s/s, p/p). Since the serine mutant belongs to variety 9 and the pyridoxine mutant belongs to variety 2, it was impossible to conduct breeding experiments to determine the presence or absence of linkage between the two genes.

Breeding experiments with each of these mutants through three generations and with appropriate test crosses resulted in ratios which support a single-gene hypothesis, although closely linked genes may well exist. The ratios were invariably skewed toward the heterozygotes, indicating a selection against the homozygotes. This situation may account for the rarity of recessive homozygotes in nature. These data, together with the mating types studies, support the idea of an outbreeding economy in *T. pyriformis*.

In an effort to gain a little more information as to the differences which apparently exist in the metabolism of the serine requiring and nonrequiring strains, C¹⁴-labeled glucose, serine, and glycine were offered to the growing cells (24a). Following a three-day incubation period, acid hydrolysates of the organisms were chromatographed and autoradiographs were made from the chromatograms. Both clones synthesized equal amounts of serine from the labeled glucose. Likewise, they both handled the labeled serine and glycine in the same way. Then why is exogenous serine required by the wild-type and not by the mutant? This puzzling question remains to be answered.

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PHYSIOLOGY AND DEVELOPMENT OF LOWER FUNGI (PHYCOMYCETES)^{1,2}

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The suggestion has often been made that most currently popular best sellers deal with religion, royalty, or sex. On the last basis at least, the aquatic Phycomycetes enjoy potential claim to fame. In their reproductive habits they display some of the most primitive, the most remarkably diverse and, indeed, the most remarkably perverse activities known to microbiology. As a consequence, many are the mycologists who have become intensely intrigued by these mysterious behavior patterns of aquatic fungi and, so to speak, how they got that way. In recent years, a beginning has been made in evaluating some of the mechanisms involved. In the following pages we shall attempt to summarize what we know of the relation of physiology to (a) phylogenetic development, and (b) certain aspects of ontogenetic development in the aquatic Phycomycetes and, to a limited extent, the terrestrial Phycomycetes.

Physiology and Phylogenetic Development in Aquatic Phycomycetes

In 1950 (1) and again in 1955 (2), attempts were made to utilize nutritional and biochemical parameters for the interpretation of evolutionary development and phylogenetic relationships among the aquatic fungi. It seems reasonable, in fact, essential to assume that real insight on evolutionary tendencies among any group of organisms will be impossible without a conscious recognition that evolution of form and structure on the one hand, and evolution of those limiting metabolic processes immediately responsible for such form and structure on the other, had to occur simultaneously. While it is probably impossible for us to determine at this time what relative weight should be placed upon our currently imperfect and incomplete picture of physiological criteria, it remains (we think) axiomatic that no valid scheme is possible if one set of parameters is used while the other set is disregarded. In

¹ The survey of the literature pertaining to this review was concluded in December, 1958.

² The following abbreviations will be used: PAB (p-aminobenzoic acid); DPN (diphosphopyridine nucleotide); TPN (triphosphopyridine nucleotide); RNA (ribonucleic acid); OC (ordinary, colorless); RS (resistant sporangium).

³ Paper #58-34 from the Department of Botany and Plant Pathology, Michigan State University.

1955 (2), the available data on the nutrition of aquatic fungi led to the following conclusions:

- 1. Among uniflagellate Phycomycetes, the Chytridiales appeared to be the most primitive because: (a) they retained the capacity to reduce sulfate and to use it as the sole source of sulfur for growth; (b) some of them retained the capacity to synthesize all vitamins essential for growth, while others had lost the capacity to synthesize one vitamin, thiamine; and (c) some retained the capacity to utilize inorganic nitrogen compounds (nitrate and ammonium salts) as sole sources of nitrogen for growth, although others apparently had lost the capacity to utilize the more highly oxidized forms such as nitrates.
- 2. The Blastocladiales appeared to be more advanced than the Chytridiales because (a) all of them had lost the capacity to utilize sulfate as the sole source of sulfur for growth; thus they were forced to make use of organic sources of sulfur. Indeed, some members of the group had progressed even further in their losses of synthetic capacity and were unable to synthesize methionine from any source, organic or inorganic; (b) all of them had lost the capacity, still retained by some of the Chytridiales, to synthesize thiamine. In addition, one member of the group had also lost the capacity to synthesize biotin and nicotinamide; (c) finally, all of them had lost the capacity, also retained by some of the Chytridiales, to utilize nitrate-N. In fact, one member of the group had even lost the capacity, retained by all the Chytridiales, to make use of ammonium-N for growth.

Thus, as far as synthetic capacities were concerned, it seemed as if the Blastocladiales were consistently more specialized and more dependent upon the environment, and thus more advanced in the sense of Lwoff (3), than the Chytridiales.

3. As for the biflagellate series of aquatic fungi, the Saprolegniales appeared to have: (a) lost the capacity to utilize sulfate-S for growth; (b) lost the capacity to utilize nitrate-N but retained the ability to use ammonium-N for growth; and (c) retained the ability to synthesize all essential vitamins.

4. The saprophytic Peronosporales, like the Saprolegniales, apparently had lost the capacity to utilize nitrate-N but retained the capacity to use ammonium-N for growth. Furthermore, they lost the capacity, which the Saprolegniales retained, to synthesize one vitamin, thiamine. On the other hand, they differed from the Saprolegniales in that they retained the ability to utilize sulfate-S.

5. Finally, the Leptomitales: (a) like the Saprolegniales but unlike the Peronosporales, had retained the capacity to synthesize all essential vitamins; however, (b) like the aquatic Peronosporales but unlike the Saprolegniales, retained the capacity to use sulfate-S for growth; and finally (c) unlike both the Saprolegniales and Peronosporales, appeared to have lost the capacity to use inorganic nitrogen compounds (nitrates and ammonium compounds) for growth.

As a consequence, and placing the emphasis on sulfur nutrition (1, 2) it

was assumed that the evolution of synthetic capacities dichotomized at some point into two main branches, one of which led to the synthetic capacities found in modern day Saprolegniales; the other was assumed to have led, by way of a subsequent dichotomy, to the two, distinct, nutritional patterns characteristic of today's Leptomitales and saprophytic Peronosporales.

Finally, the general picture as reviewed in 1955 (2) suggests that the uniflagellate series of lower fungi was characterized by a predominantly fermentative type of metabolism; the biflagellate series, a predominantly oxidative type. Since 1955, new studies have appeared which bear upon this subject; they are itemized below.

Chytridiales.—Investigations (4) of the nutritional requirements of Phlyctorhiza variabilis provide three important points that bear upon our story: (a) Phlyctorhiza can utilize inorganic nitrogen (ammonium compounds) as the sole source of nitrogen for growth, although other organic nitrogen compounds such as amino acids appear to be superior in this respect. (b) It requires an exogenous supply of both biotin and nicotinamide for growth. And (c) it grows vigorously under fairly low (microaerophilic) oxygen tensions, but not under strictly anaerobic conditions.

Blastocladiales.—In a study of the comparative nutrition of two species of Blastocladia, Craseman has found (5) that; (a) B. ramosa and B. pringsheimii can use ammonium-N, but not nitrate-N, as the sole source of nitrogen for growth. (b) Both B. ramosa and B. pringshemii require exogenous supplies of thiamine and nicotinamide for growth. In addition, her strain of B. pringsheimii, unlike Cantino's (6), did not require biotin but did require PAB, while B. ramosa did not require either biotin or PAB. (c) Neither species of Blastocladia was able to use sulfate-S; instead, they required organic-sulfur compounds. B. ramosa used methionine, cysteine, and cystine, but B. pringsheimii apparently had an absolute requirement for methionine. Finally, (d) B. ramosa produced lactic acid and, in fact, grew vigorously under anaerobic conditions.

Comparative studies of both gametophytic and sporophytic cultures of Allomyces (7) reveal that the nutritional requirements, growth rates (except for differences in lag periods), and certain aspects of carbon and nitrogen nutrition of the gametophytes of Allomyces are essentially identical to those of the sporophytes. On the other hand, respiratory activity of the 2N sporophyte is about one-half again as high as that of the 1N gametophyte of comparable physiological age, although sensitivity to arsenite inhibition is about the same for both (8). With respect to the sporophyte in particular, knowledge of Allomyces has been extended beyond that provided by Ingraham & Emerson (9), who had shown that (a) it was an obligate aerobe which, under aerobic conditions, converted approximately one-third of glucose carbon to lactic acid, one-third to carbon dioxide, and one-third to cell material; but which (b) under anaerobic conditions, metabolized glucose without production of carbon dioxide to yield lactic acid in an almost-pure homofermentative type of dissimilation. Apparently, however, Allomyces may also liberate

other organic acids (8); it has been reported that a metabolic product with the chromatographic characteristics of oxalic acid, and that traces of pyruvic acid are detectable in filtrates of both gametophytic and sporophytic cultures of Allomyces grown on synthetic media. Although most intermediates of the tricarboxylic acid cycle do not serve as carbon sources for growth of Allomyces, acetate does seem to serve in this capacity (10). Furthermore, while total absence of carbon dioxide brings about decreased growth of Allomyces (11), this effect is overcome by certain concentrations of glutamate. Bonner & Machlis (12) provide additional details about respiration in the sporophyte of Allomyces. Metabolism of labeled glucose by intact plants yields very little radioactivity in tricarboxylic acid cycle intermediates; at the same time, the increased oxygen consumption brought about by glucose is inhibited by iodoacetate, fluoride, fluoroacetate, and arsenite, while the increase in oxygen consumption brought about by acetate is not inhibited by iodoacetate. These findings are consistent with earlier observations already cited (2) that, under aerobic conditions, Allomyces may produce very little lactic acid from glucose, while that portion of the glucose not assimilated (9) may be oxidized almost completely to carbon dioxide (10). Mitochondrial preparations from Allomyces oxidize almost all intermediates of the tricarboxylic acid cycle; oxidation of succinate was malonate-sensitive (12). Thus, the evidence suggests that, given sufficient oxygen, Allomyces may metabolize glucose by way of the glycolytic pathway, through the tricarboxylic acid cycle, to carbon dioxide.

For Blastocladiella, on the other hand, the most recent data (13) corroborate earlier notions (2) that metabolism is primarily fermentative rather than oxidative.

Saprolegniales.—Other than three fascinating reports by Fischer & Werner (14, 15, 16) on (a) the effect of amino acids and salts upon directional growth and branching of the mycelium of Saprolegnia, and (b) the paralytic effect of nicotinamide upon the flagella of the Saprolegniales, no additional pertinent information is available on the nutrition and metabolism of this group.

Leptomitales.—Goluecke (17) reports on a comparative study of the physiology of Sapromyces, Rhipidium, and Apodachlya —members of the Leptomitales. With respect to vitamin nutrition, Sapromyces requires an exogenous supply of thiamine, while Apodachyla requires none. With Rhipidium, the results were inconclusive; but probably, it also is autotrophic for all vitamins. Both Sapromyces and Apodachlya are able to use a variety of tricarboxylic acid cycle intermediates as carbon sources for growth; neither one can grow under anaerobic conditions, and both grew much better under conditions of forced aeration than in stationary cultures. Neither Sapromyces, Rhipidium, nor Apodachlya could use nitrate or ammonium compounds as sole sources of nitrogen for growth. However, all three were apparently able to reduce sulfate and use it as a sulfur source for growth. With an ample

supply of oxygen, Sapromyces and Rhipidium dissimilated glucose completely to carbon dioxide with no appreciable accumulation of acids, while under anaerobic conditions, both appeared to convert glucose stoichiometrically to lactic acid.

One other report on the nutrition of the Leptomitales has appeared (18) which purports to show that Apodachyla can use either nitrate or ammonium compounds as sole sources of nitrogen for growth. Because of the obvious unreliability of the methods used to evaluate growth responses, the lack of adequate pH control, the lack of sufficient controls for studying N-nutrition (e.g., controls lacking a nitrogen source), etc., the results reported in this paper are seriously open to question.

Peronosporales (Pythiaceae).-Lopatecki & Newton (19) report on the nitrogen and vitamin nutrition of four species of Phytophthora. There seems to be no question that here, too, an exogenous supply of thiamine is needed for growth. But, with respect to nitrogen nutrition, it is somewhat difficult to evaluate the results. The authors conclude that three of the four species grow well with ammonium-N, while the fourth (P. megasperma) grows poorly; on the other hand, Phytophthora cactorum seems to grow poorly on nitrate-N while the others grow well, Unfortunately, the lack of adequate controls renders the results open to some question. Nevertheless, it is beginning to appear as if nitrate-N may be utilized by some members of the Pythiaceae [see also, Wills (20) on P. parasitica]. Indeed, M. E. Davies (personal communication) reports that, according to Dr. Fleetwood Walker, and contrary to the results of Lopatecki & Newton (19), P. cactorum can assimilate nitrate; Christie (20a) reports similarly. On the other hand, Davies believes that nitrate and ammonium salts are not assimilated by P. fragariae even in the presence of organic acids over a wide pH range, while ammonium salts are used by P. erythroseptica in the presence of organic acids.

Bitancourt et al. (21) report again on the essentiality of thiamine for branching in *Phytophthora*. Sakai (22) also finds that *Phytophthora infestans* requires thiamine for growth. He concludes that inorganic nitrogen compounds such as nitrates, and ammonium salts may support growth. Here, too, more carefully controlled experiments are in order, but we are left once again with the suggestion that nitrate-N may suffice to support the growth of this strain of *Phytophthora*. The same author (23) also reports that succinic and oxalic acids are products of the metabolism of *Phytophthora*.

Finally, these considerations of recent studies of the Leptomitales and Peronosporales lead logically to the recognition that there is continued need for a greater awareness of the pitfalls to be avoided in nutritional studies (e.g., the difference in response of fungi to different optical isomers, as illustrated by Newton's (24) report of the ineffectiveness of D-alanine as a nitrogen source for *Phytophthora* as compared to L-alanine; the effects of autoclaving on nutritional studies, such as McKeen's (25) observations that glycine serves as a fine nitrogen source for *Phytophthora fragariae*, but sup-



ports no growth at all if it has been autoclaved with glucose in the growth medium; and, in general, more critical and more carefully controlled approaches (2) to nutritional experiments).

Conclusions on the relationship between nutrition and phylogeny.—No matter how objective the approach, any consideration of the relation between physiology and phylogeny carries with it some degree of speculation and the need for a tentative acceptance of certain working principles upon which some sort of superstructure can be based. In this particular case, for example, tentative acceptance of two important building blocks is essential:

First, that physiological evolution, in the sense of Lwoff (3), may lead to progressive losses in capacity for synthesis (heterotrophism) and thus to a progressively increasing dependence of the organisms upon the environment. Therefore, in contrast to the apparent general course of evolution leading toward progressive specialization and an increase in morphological complexity, we deal here with a gradual decrease in complexity of metabolic machinery.

Second, that in this sort of a localized evolutionary chain, the losses are progressive and cumulative; that is, that a loss in capacity for synthesis is not regained, and that all such losses experienced by a progenitor will be felt similarly by its progeny.

Within this framework, the picture for aquatic fungi becomes reasonably clear (Fig. 1). The fact that there existed interordinal differences in the synthetic capacities of aquatic Phycomycetes was obvious in 1955 (2); the most recent studies corroborate this conclusion. In addition, the work of the past few years which has been reviewed herein extends and further emphasizes the conclusions reached in 1955; e.g., (a) in the uniflagellate series, the nutritional characteristics of the Blastocladiales are more advanced than those of the Chytridiales from which they seem to have evolved by progressive losses in synthetic capacity; and (b) in the biflagellate series, the degree of heterotrophy and the nature of the specific nutritional requirements of the Leptomitales and Peronosporales seem to relate these two orders more closely to one another than to the Saprolegniales.

Finally, it may even be possible to derive predictive value from the scheme presented in Figure 1. On morphological grounds, the Monoble-pharidales probably evolved more or less directly from the Blastocladiales; at least, the latter appear to be the most likely progenitors. As yet, however, no one has succeeded in establishing their nutritional requirements. Therefore, employing the scheme in Figure 1, we would consider it a good risk to predict that all the Monoblepharidales will be found to have lost the capacity (a) to synthesize thiamine and (b) to use nitrate and sulfate compounds as sole sources of nitrogen and sulfur, respectively; and that at least some of the members of the group may even have lost the capacity (c) to use ammonium compounds as sole sources of nitrogen for growth, (d) to synthesize some essential organic sulfur compound, and (e) to synthesize vitamins other

than thiamine. Indeed, these may be the very reasons why the Monoblepharidales have not as yet been cultured in synthetic media!

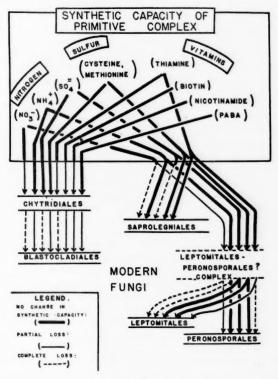


Fig. 1

Physiology and Ontogenetic Development in Aquatic Phycomycetes

Not long ago, Stetten (26) wrote, "The investigator may elect to employ the minimally disturbed intact animal, the isolated perfused organ or tissue of that animal, the sliced, teased, or minced preparation of such a tissue, the cell-free homogenate, or the solution of enzyme or enzymes. Each level as listed here represents a further degree of disorganization of an initially highly integrated system and the disorganization is in each case imposed by the investigator. At each successive level of disorganization, something is gained in the form of greater simplicity, greater control over variables, greater faculty in establishing the necessary components of the system for

reaction under scrutiny. However, at each successive level of disorganization, something of importance is also lost." Although Stetten's remarks dealt with the biochemistry of disease, he has emphasized, as lucidly and as forcefully as anyone who has expressed a similar conviction, the complications which confront all of us who wish to attack the profound riddle of differentiation in plants and animals-and, of course, it applies to the lower fungi to which this chapter is devoted. It is understandable-indeed, inevitable-that opinions on this obscure and baffling phenomenon, fortified by various degrees of emotional potential, have been driven over a broad range: opinions about the relative merits of the "descriptive," the "experimental," the "philosophical" approaches toward a solution of the problem, the methods which can be expected to yield the greatest reward, the question of what it is that is being sought, and last, but hardly least of all, the judgement and interpretation of the results obtained therefrom and their judicious utilization for the creation of theories and doctrines of morphogenesis. There are many among those comprising the school of thought who deplore the "present accelerating trend toward the cell homogenizer by workers who are rapidly losing sight of the ultimate reasons for studying the soups and breis which at present hold their attention" (27). That the trend exists is probably true even for the lower fungi-but that this reflects a rapid loss of sight is vigorously contested by numerous converts to the even larger school of cell homogenizers. And yet, these two rival schools in turn find strength and common bonding in their often equally spirited convictions that some (or all) other camps of thought cannot be further from the truth. Like the three blind wisemen, each of whom was sure he had deduced the nature of the protoplast, it seems likely that each school of thought is partly in the right, yet all of them are wrong. As in the "No Man's Land between the autotrophic and heterotrophic ways of life" (28) but on a scale of far greater dimensions, the area between apparent morphology at the organismal or cellular level, and submicroscopic, biochemical, and biophysical phenomena found therein at a dynamic molecular level, is one replete with dichotomies and anastomoses at every conceivable level of integration. The degrees of complexity seriously stagger the imagination.

It is no wonder, therefore, that attempts to understand the mechanism of morphogenesis in aquatic fungi have taken many diverse pathways, e.g.: (a) the numerous morphological, anatomical, histological, and generally "descriptive" approaches to an understanding of differentiation and concomitantly, the objections from different quarters that such approaches have not led to generally acceptable doctrines of differentiation based upon the more fundamental (so some say) underlying biochemical phenomena which presumably motivate differentiation. (b) Attempts to correlate ontogenetic events with external and environmental variables. (c) The very few attempts, based in part upon the foregoing sorts of approaches, to synthesize working hypotheses at physiological levels of integration (e.g., hormonal mechanisms involving sex hormones and growth substances which

directly or indirectly mediate the inception or formation of differentiated cells, tissues, and "organs"); and, along with this, the gradually-accumulating awareness that it may be essential to distinguish clearly between mechanisms for initiating differentiation and mechanisms for perpetuating it. And finally, (d) scattered attempts to find alterations in internal composition and metabolic activity associated with changes in cellular and organismal morphology; and, admonitions from some quarters here, too, that such approaches are essentially "descriptive" and have limited utility for explaining morphogenesis if they are not supplemented with the experimental manipulation of normal developmental patterns.

And yet, although differences of opinion probably outnumber the manifold attacks on this problem, there is one note of harmony. Almost all mycologists, will probably agree (though some reluctantly) that the end in view is to attempt to place equitably the relative morphogenetic roles of cytoplasm and nucleus, internal and external environment, genetic change and enzymatic adaptation, all in their proper perspective—an eminently worthy but Herculean task. And almost all will also agree that for the lower fungi, to which this chapter is dedicated, our knowledge of the dynamics of morphogenesis in physiological and biochemical terms is at most very,

very fragmentary.

The writers have exercised their prerogative and have chosen to confine this section of the review to those very few areas in the experimental study of aquatic fungi where a beginning has been made in relating (a) the mechanism of asexual morphogenesis in an apparently cause-and-effect fashion to specific biochemical events, and (b) biochemical and cytochemical factors to sexual morphogenesis. Within such parameters, the number of papers which begin to qualify is small, indeed. The important and fascinating Achlya story (29) which is well known and has been extensively reviewed elsewhere, and studies of the hormonal control of sexual reproduction in other Saprolegniales and the Peronosporales [(30, 87a) and references therein], are not included.

ASEXUAL DIFFERENTIATION IN AQUATIC FUNGI; THE FORMATION OF RESISTANT SPORANGIA IN THE BLASTOCLADIALES

In the life history of these fungi, a motile, uniflagellate, uninucleate swarmer germinates in bipolar fashion to produce a multinucleate thallus bearing a basal rhizoidal system. In Allomyces, the thallus becomes an extensively-branched myclelium which, depending upon the type of swarmer used, may bear: (a) paired, differentiated, male and female gametangia; or (b) isolated, thin-walled, colorless zoosporangia (mitosporangia) and brown, thick-walled, pitted, resistant sporangia (meiosporangia). In Blastocladia and Blastocladiella, on the other hand, the thallus is not mycelial, but displays a determinate system of growth. In Blastocladia, the thallus is usually branched and may bear both thin-walled, colorless zoosporangia and the brown, pitted, resistant sporangia. In Blastocladiella, the thallus is not

branched. As the thallus matures, a septum is laid down which separates the plant into two cells, a basal, rhizoidal cell and a terminal, globose one. In this case, the terminal cell may be either a thin-walled, colorless one or a brown, thick-walled, resistant sporangium.

The main criterion which pinpoints the Blastocladiales taxonomically is the production of this brown, thick-walled, normally-pitted, resistant sporangium [cf. Sparrow (31)]. In recent years, intensive experimental studies on the general biology of these fungi have been accumulating rapidly [Emerson (32)]; among them, studies of the differentiation of resistant sporangia have led to some promising notions of the biochemical mechanisms which motivate morphogenesis in Blastocladiella.

which motivate morphogenesis in Blastocladiella.

Blastocladiella.—In essence, the problem is this: until about three-fifths of the generation time of the plant has elapsed (33), development may occur along either of two major morphogenetic pathways (34). The terminal cell at maturity may be thin-walled, colorless, and bear prominent discharge papillae through which thousands of uniflagellate swarmers are ultimately discharged through pores formed by the deliquescence of these papillae. This cell may be called an ordinary, colorless, (OC) cell. Thus, the OC type of plant functions to propagate continuously, ordinary vegetative growth of plastocladiella. On the other hand, the terminal cell may look distinctly different and possess a very thick, brown, pitted wall devoid of discharge papillae. This structure is the resistant sporangium (RS); it usually does not germinate immediately to function in the continuous propagation of the fungus unless it is transferred to a new environment.

When Blastocladiella emersonii is found on natural substrata submerged in a body of fresh water such as a pond, it may develop along one morphogenetic pathway or the other. The obvious question is: what are the factors

which control these developmental pathways?

The nature of colorless OC plants.—Actively proliferating OC plants seem to carry on a predominantly homofermentative type of metabolism, whereby two moles of lactic acid are recovered for each mole of glucose consumed (35). The net output of carbon dioxide during active growth is detectable but is very low; the average Q_{CO2} is less than 0.1 (13, 35). When nonproliferating plants dissimilate labeled glucose, the only radioactive, nonvolatile acid (other than amino acids) recovered after periods up to 1 hr. is lactic acid (36). Also, with preformed plants, glucose does not induce increased oxygen consumption over the endogenous Qo2 of 9.0, and no detectable carbon dioxide is produced under these conditions (37). Cell-free preparations of OC plants exhibit most of the enzymatic activities associated with the glycolytic pathway leading from hexose phosphate, through exclusively DPN-specific reactions, to pyruvic and lactic acids (13). Thus, glucose metabolism in both proliferating and nonproliferating OC plants appeared to be mainly fermentative and, at the very most, weakly oxidative. On the other hand, enzymatic and chemical assays have led to the conclusion (13, 38, 39, 40) that the tricarboxylic acid cycle in OC plants is at least potentially operative, but that the forces which drive or pull it, or the rate limiting steps within it or both, make it a feeble and weakly functional system. Therefore, it can play no more than a minor, quantitative role in supplying energy for growth and for maintenance; on the other hand, the enzymatic activities found therein probably play vital roles elsewhere, one of which is to help mediate carbon dioxide fixation (13).

The morphogenetic shift from proliferating OC plants to nonproliferating RS plants.—Virtually all the members of a population of Blastocladiella will cease to proliferate by way of OC plants and, instead, will form the RS plant when bicarbonate is incorporated in a suitable growth medium under otherwise essentially identical (34) conditions. Up to three-fifths of the generation time of a plant, the bicarbonate trigger mechanism operates; beyond this time, however, the morphogenetic pattern cannot be altered by this concentration of bicarbonate, and only OC plants are produced. Because a gradually decreasing permeability seems to be involved during the genesis of an RS plant (33), this critical period in ontogeny fluctuates with the concentration of bicarbonate used. But, at any rate, the product is a resistant sporangium which does not proliferate further under these conditions.

The nature of the RS plants produced in response to bicarbonate.—The RS plant differs qualitatively from an OC plant as follows: (a) its wall is impregnated with melanin and an active polyphenol oxidase (41), and (b) its protoplast contains γ -carotene (36) and a particular, electrophoretically-separable, protein fraction (13, 42); none of these is found in OC plants.

The RS plant differs quantitatively from an OC plant as follows: on the credit side, its wall contains more chitin (43) and its protoplast contains a greater pool of ketoglutarate (13, 34) and lipoidal material than OC plants; on the debit side, its protoplast contains a much lower pool of free amino acids, total acid soluble nitrogen, chitinase, and enzymes concerned with glyoxylate metabolism (43, 44); it has suffered an almost complete loss of tricarboxylic acid cycle enzymes (except a TPN-specific isocitric dehydrogenase), a terminal cytochrome oxidase, and two, electrophoretically-separable protein fractions (13, 34, 41) found in OC plants; and finally, the over-all metabolic machine has a much reduced oxygen consumption as compared to OC plants (43). Thus, the bicarbonate trigger mechanism has induced a severe alteration of the intracellular nitrogen pool resulting in decreased enzyme activity and endogenous respiration, and at the same time, increased synthesis of chitin and a de novo synthesis of melanin, carotene, and at least one new protein fraction.

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Mechanism and coupling reactions.—At a biochemical level of integration, a notion has been developed regarding the way in which the bicarbonate trigger mechanism may operate. Bicarbonate seems to interfere with oxidative decarboxylations in the weakly functional tricarboxylic acid cycle. The flow of intermediates along this part of the cycle is stopped and then reversed. The accumulation of ketoglutarate, its reductive carboxylation, and the products resulting therefrom constitute a metabolic shift which leads to the genesis of a resistant sporangium. Such a mechanism need not interfere with the energy needs of the developing organism because the bulk of its requirement is normally derived from fermentative metabolism.

Perhaps the most crucial part of the story of morphogenesis in Blastocladiella centers upon the way in which the reducing power to drive the reductive carboxylations is generated, and the methods by which the carboxylations are coupled to biosynthetic reactions leading to the synthesis of the chitin, fat, melanin, carotene, and other components associated with

the structure and function of a resistant sporangium.

With respect to melanin, which appears de novo during genesis of a resistant sporangium but which does not occur in an OC plant, the evidence available provides a possible mechanism by which the bicarbonate effect is coupled to melanin production. In vivo, one can interfere selectively with the synthesis of melanin without interfering with the production of an otherwise normal resistant sporangium by incorporating phenylthiourea in the medium during growth (39). Thus, it is possible to uncouple from the bicarbonate trigger mechanism, one of the reactions induced by it without interfering with other events simultaneously motivated by bicarbonate. The sudden appearance of melanin in a resistant sporangium is associated with the de novo appearance of a phenylthiourea-sensitive polyphenol oxidase not present in OC plants (41). Also, in the pool of amino acids which drop so sharply in concentration during the genesis of the RS plant, tyrosine (established substrate for the polyphenol oxidase in Blastocladiella, and presumed substrate (41) for melanogenesis) displays the most striking reduction (43). Furthermore, electron transport from the polyphenol oxidase system is coupled to the reduction of TPN (but not DPN), thus establishing an important link with the TPN-specific reductive carboxylation of ketoglutarate to isocitrate by way of an isocitric dehydrogenase which remains functional during the formation of an RS plant (41). Isocitritase is present in Blastocladiella (44) and presumably helps to mediate the removal of isocitric acid from the site of action. As for the rest of the story on morphogenesis, efforts are being made to determine (13, 42) if the reductive carboxylation of ketoglutarate and products derived directly therefrom, or for that matter the bicarbonate itself, are directly or indirectly coupled to the decrease in chitinase activity, amino acid pools, and protein pools, and the increased chitin synthesis which occur during differentiation of an RS plant. Such coupling must exist, however, even though it may be far removed from the reductive carboxylation site and may involve a network of many reactions rather than one or two primary ones. Hopefully, they will be found and their role in morphogenesis established.

Summary.—The picture of the biochemical basis for morphogenesis in Blastocladiella as seen at this time is as follows:

Bicarbonate interferes with, slows down and finally stops a weakly functional tricarboxylic cycle in young germlings and young OC plants which

have not progressed beyond three-fifths of their generation time; the loci for the effect are the two, successive, reductive, carboxylation sites. Carbon dioxide fixation via pyruvate or phosphoenolpyruvate seems unlikely (13).

As this happens, the DPN-specific enzyme activities and the other nonnucleotide dependent reactions of the tricarboxylic acid cycle as well as a terminal cytochrome oxidase, are destroyed, inactivated, or in some other way rendered inoperative. But, the TPN-specific isocitric dehydrogenase activity remains fully functional, and with the disappearance of these enzyme systems it begins to mediate with increasing effectiveness a TPN-specific reductive carboxylation of ketoglutarate. Part of the required reducing power may arise by way of the potent TPN-specific glucose-6-phosphate and 6-phosphogluconic dehydrogenase (13, 42, 45) found in OC plants, and part of it may be supplied by radiant energy if plants are light-grown (36, 45, 46).

At the same time, the tyrosine of the amino acid pool is funneled into the machinery for melanin synthesis. An oxygen-independent polyphenol oxidase appears whose activity is coupled by way of TPN-reduction to—and, indeed, enchanced by—the TPN-specific reductive carboxylation of ketoglutarate which regenerates the oxidized nucleotide. The TPN-specific glucose-6-phosphate dehydrogenase mentioned above provides additional reducing power to drive the reductive carboxylation and, simultaneously, formation of oxidized nucleotide for the polyphenol oxidase which, in turn, helps to manufacture the melanin of the RS plant.

These reactions, once under way, are given a gradually increasing boost by a gradually decreasing permeability of the cell to bicarbonate (thus leading to increased retention of exogenously supplied bicarbonate or metabolically generated bicarbonate or both) as morphogenesis progresses.

The end result is a thick-walled, brown, resistant sporangium, devoid of a functional tricarboxylic acid cycle and with very low oxidative activity, very resistant to and well-equipped to stand adverse environmental conditions, but with the built-in potentiality of regenerating all that it has lost and losing all that it has gained once the second generation swarmers are liberated to repeat the cycle. A detailed discussion of the data upon which these conclusions are based is provided by Cantino (13).

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Recently, Jaffe (47) had occasion to remark briefly upon these studies of morphogenesis in Blastocladiella. After obviously misreading the first paper on this fungus (35) and attributing to it a characteristic it does not possess ("... producing no net CO₂ at all."), he suggested that bicarbonate acts by simply lowering the extracellular or intracellular pH, or both. Jaffe overlooked the direct evidence that: (a) bicarbonate causes a slight increase in internal pH (33); (b) bicarbonate causes a slight increase in the external pH (34); but (c) most important, aside from the direction of a pH shift, manipulation of the external pH in the absence of bicarbonate does not induce formation of resistant sporangia [(34); also, see comments in the following section on Blastocladia]. Finally, Jaffe seems to have overlooked all of the

data which appeared after 1951 which strongly support Cantino's (13) point of view and which cannot be explained away by the magic of pH!

Blastocladia.—In the family Blastocladiacease, there occurs a second genus, Blastocladia. Some aspects of our more limited knowledge of the physiology of Blastocladia bear upon the morphogenetic mechanisms in Blastocladiella and, in fact, suggest that the biochemical basis for the genesis of resistant sporangia may be the same for both genera.

Like other members of the group, Blastocladia produces brown, pitted. thick-walled resistant sporangia. Although the pigment has not been analyzed chemically, it is probably a good guess that it is melanin, Microchemical tests (48) suggest that the wall contains chitin but not cellulose. On all media tested to date, Blastocladia pringsheimii produces resistant sporangia only if it is provided with a carbonic acid-bicarbonate system at the correct pH (49, 50). As is the case for Blastocladiella, Blastocladia does not respond to adjustments in pH in the absence of added bicarbonate, carbonate or carbon dioxide (50). Some strains of Blastocladia will grow under conditions of almost complete anaerobiosis; others, at least microaerophilically (5, 49, 50). Blastocladia also produces lactic acid copiously when it dissimilates glucose, some 85 per cent or more of the glucose-carbon being recovered as lactic acid and 11 per cent as succinic acid, whether or not conditions are aerobic or anaerobic; furthermore, it produces no detectable carbon dioxide during growth or during glucose dissimilation in the absence of growth (50). Thus, like Blastocladiella, it appears to carry on a fermentative type of metabolism with a strong tendency for formation of lactic acid.

In addition, when Blastocladia dissimilates glucose in the carbonic-bicarbonate system which leads to the genesis of resistant sporangia, (a) the amount of glucose-carbon recovered as lactic acid is reduced from 85 to 90 per cent to about 75 per cent, while the amount recovered as succinic acid is almost doubled as it increases from about 11 per cent to 20 per cent; and (b) the recovery of glucose-carbon as lactic and succinic acids is reduced from an average of 97 per cent in the absence of carbon dioxide-bicarbonate media to an average of 94 per cent in their presence (50). This response is reminiscent of the increase in labeled succinate which occurs in Blastocladiella following increased rates of CO₂ fixation (36). The available facts point strongly to the likelihood that the biochemical basis for morphogenesis in Blastocladia is closely related to that which seems to operate in Blastocladiella.

Allomyces; R. S. formation in the sporophyte.—In contrast to the situation in Blastocladiella and Blastocladia, our knowledge of the relationship between physiology and formation of resistant sporangia (meiosporangia) in Allomyces is still scanty. In general, no special treatment is required to induce genesis of resistant sporangia when the diploid sporophyte is grown on Emerson's (49) YpSs medium; they are usually formed in abundance shortly after, and sometimes simultaneous with, production of the colorless, thin-walled, zoosporangia (mitosporangia). While temperature may exert some control (51), and certain media [e.g., Sost's (52) peptone medium] may be less favor-

able for genesis of resistant sporangia than zoosporangia, these observations do not provide insight into the basic relation between physiology and morphogenesis. One interesting observation is worth noting, however. Turian & Chodat (8) report that arsenite tends to induce formation of resistant sporangia with varying degrees of wall thickness and pitting in the sporophytes of Allomyces incubated in saline solution, while control plants produce only zoosporangia under these same conditions. It calls to mind the fact that arsenite also induces RS formation (35) and prevents RS germination (39) in Blastocladiella, presumably by interfering with oxidative decarboxylation of ketoglutarate (38); it begins to look as if the biochemical basis for morphogenesis in these two genera may also have a common basis.

RS formation in the gametophyte.—Manipulation of certain environmental conditions (53) leads to differentiation of resistant sporangia on the haploid gametophyte of Allomyces during growth on Emerson's YpSs (49) medium. Of particular interest is the observation that subtoxic concentrations (10⁻⁸ M) of cupric ion lead to the formation of brownish colonies rather than the usual orange ones. Microscopic observations suggest that copper brings about (a) increased differentiation of male gametangia; (b) production of structures of uncertain identity which are difficult to categorize because they contain the orange pigment of male sex organs but also possess thickened walls with various degrees of pitting; and (c) genesis of typical, deep brown, normally-pitted, resistant sporangia. In view of the presence of polyphenol oxidase in Blastocladiella (41), its role in RS formation, and its susceptibility to copper-enzyme inhibitors in vitro (41) and in vivo (39), perhaps the treatment with copper has simply provided an essential, limiting component for polyphenol oxidase activity in Allomyces gametophytes which, for some reason, may be normally deficient in their internal supply of an adequate soluble pool of available copper for this purpose. Arsenite, too (54), induces occasional differentiation of resistant sporangia on gametangial hyphae in liquid culture as it does for the sporophyte (see comments, above). In addition, gametophyte explants, when transferred to nitrogen-depleted, synthetic media wherein they grow but slightly, produce resistant sporangia in close proximity to normal pairs of male and female gametangia (53).

Finally, concerning the composition of the resistant sporangia of Allomyces, nothing is known of their enzymatic makeup. The wall material, which probably contains chitin (48), is impregnated with melanin (55). In the protoplast are found transitory chromospheres which contain RNA (56, 57); Rorem & Machlis summarize current speculations regarding the function of these organelles (57). Turian & Haxo (58) reported that sporo-

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⁴ Stumm (57a) has induced formation of resistant sporangia in *Allomyces* with x-rays and has studied these structures cytologically; he believes that such RS are not the seat of meiosis as is the case for normal, 2N, resistant sporangia and that, instead, germination and formation of RS spores results from a mitotic process. Thus, Stumm's x-ray-induced RS are presumed to be haploid. It seems unfortunate that Stumm did not solidify his conclusion with (a) a search for the presence or absence

phytes of Allomyces bearing resistant sporangia appeared to be devoid of both colorless and colored polyenes. This conclusion seems to contrast sharply with the situation in Blastocladiella, where easily detectable amounts of γ -carotene are present in resistant sporangia (36). However, while the techniques used probably insured fragmentation of the zoosporangia and mycelium of the sporophyte, it is quite possible that most of the thick-walled, resistant sporangia may not have suffered the same fate. Like the resistant sporangia of Blastocladiella which are almost impossible to break when homogenization procedures not involving abrasives are used (41), the RS of Allomyces may well have remained intact and unextractable. Therefore, it remains a distinct possibility that the RS of Allomyces may, indeed, contain carotenoids. Turian's observations (see above) on the production of yellow-colored resistant sporangia, intermediate in character between typically-orange, male gametangia and normally-brown, resistant sporangia, tend to support this point of view. In fact, these "hybrid" structures are reminiscent of the intermediate types of RS containing visible patches of orange pigment which can be found in Blastocladiella (59). At this point, there is insufficient evidence for sweeping generalizations. Nevertheless, it does seem as if the relationships between the orange, colorless, and brown cells in Blastocladiella (60, 61) and those described above for Allomyces, and the factors which motivate their formation, respectively, may have a good deal in common.

SEXUAL DIFFERENTIATION IN AQUATIC FUNGI; THE FORMATION OF SEX ORGANS IN THE BLASTOCLADIALES

This section is devoted primarily to the kind of differentiation in the haploid, sexual generation of Allomyces which gives rise to pairs of adjacent sex organs, one a bright orange, male gametangium, and the other a colorless, female, gametangium. The latter produces a diffusible, relatively stable hormone [Machlis, (61a)], apparently of relatively low molecular weight, which attracts male gametes and thus leads to gametic copulation and the formation of a diploid, nonsexual generation. An effort has been made to emphasize, where possible, apparent homologies between the situation found in sexual strains of Allomyces and the differentiation of orange and colorless cells in its "asexual" (59, 60) relative, Blastocladiella emersonii. Unfortunately, no pertinent reports have appeared regarding Blastocladiella variabilis, a species displaying true gametic copulation, since it was described and studied in detail by Harder & Sörgel (62).

Stages of sexual differentiation in Allomyces.—The sequence of morpho-

of chromospheres which are known to occur in normal, diploid RS (as mentioned above); and (b) an attempt to determine if the number of spores per sporangium was significantly less than 4 times the number of nuclei in the mature resistant sporangia before germination [as has been demonstrated by Emerson & Wilson (57b) for normal, 2N, resistant sporangia].

genetic events involved in sexual differentiation in the gametophyte of Allomyces arbuscula, subgenus Euallomyces (63), was described by Hatch (64). With lucid phase-contrast photomicrographs, Emerson (65) provided further details about gametogenesis in A. macrogynus. More recently, Turian (53, 54) made comparative cytological and cytochemical studies of sexual differentiation in the epigynous A. macrogynus and the hypogynous A. arbuscula. His previous observations that M/600 boric acid prevented gametic copulation (and slowed down germination of zygotes) provided the tool needed for propagating vegetatively gametophytic mycelium in liquid media (54a); young, undifferentiated gametophytic hyphae were then transferred to hypotonic solutions to induce sexual differentiation. Consequently, it was possible to describe the very early, less conspicuous transformations which preceded the more obvious, final phases of sexual differentiation. Three stages in development of sex organs from undifferentiated hyphal tips were recognized: (a) cytological and biochemical protoplasmic disjunction (cytochemodifferentiation), at which point the hyphal tip ceases to elongate but enlarges; (b) septation (cytodieresis), wherein one septum separates the two sexual territories from one another and a second wall separates reproductive from vegetative protoplasm; and (c) gametangial maturation. The last stage was visualized as consisting of four parts corresponding more or less to those of Hatch (64): first, cytoplasmic granulation and formation of papillae, nuclear multiplication, and (in the males) faintly yellow pigmentation; second, organization of crowns of lipide granules around the nuclei; third, organization of basophilic nuclear caps containing RNA and cleavage into gametes; and fourth, liberation of gametes.

The cytoplasmic apparatus and differentiation.—In view of Hatch's (64) comments regarding the possible role of mitochondria in sexual differentiation in A. arbuscula, the disjunction stage was subjected (53) to detailed cytological and cytochemical analysis. If apical accumulation of mitochondria in fertile hyphae [Hatch (64)] were related in cause-and-effect fashion to differentiation of female gametangia in the hypogynous A. arbuscula, it would follow that subapical accumulation of mitochondria should occur in the fertile hyphae of the epigynous A. macrogynus. But with phase-contrast microscopy, Turian (54) consistently observed that accumulation of mitochondria was apical in both species! Indeed, he concluded that such apical accumulation may be more apparent than real, and that it may, at least in part, result from fragmentation of long mitochondria into shorter rods in the tips of fertile hyphae [e.g., as has been reported in the Saprolegniales; Guilliermond et al. (65)]. Similarly, no striking differences between the two species were observed when sex organs were examined at later stages in development-e.g., in the cytoplasmic granulation stage of gametangial maturation.

However, Turian's most recent evidence (53) demands a reconsideration of the role of cytoplasmic particulates in sexual differentiation. Apparently, some of the members of a "mitochondrial" population in *Allomyces* react

strongly with the Nadi reagent while others do not, perhaps suggestive of different categories of enzymatically-active mitochondria [such as Perner's sphaerosomes (66) in higher plants. Upon reinvestigating the story on the light of these new observations, he found that the particles (identified microscopically as mitochondria) which reacted with the Nadi reagent were more numerous in the female cells than they were in the male cells! And of course, this latest description of the situation in Allomyces is highly reminiscent of the behavior patterns [Cantino & Horenstein (61)] in Blastocladiella emersonii, where a low content of Nadi-positive, cytoplasmic particles ("gamma" particles) is associated with orange ("male"?) plants, a high content with colorless ("female"?) plants. In B. emersonii, swarmers from these colorless and orange plants do not fuse with one another by conventional, irreversible, gametic copulation, but they do combine transiently via a short-lived cytoplasmic bridge (59). Crossing experiments led to the inference that cytoplasmic components such as the "gamma" particles were probably exchanged through these bridges, and that they may have been responsible for altered ratios of colorless to orange plants in subsequent generations. At the physiological level, orange and colorless plants of B. emersonii differ in enzymatic activities, nutritional requirements, and other properties (38, 40, 59, 60); it has been hypothesized that these differences may be related to the distribution of "gamma" particles. Following her recent studies (60a) of the inheritance of pigmentation using albino isolates, Foley has also suggested the possibility of cytoplasmic control of this characteristic in Allomyces arbuscula. In any case, these recent studies re-emphasize the apparent homology between colorless "sporangia" of Blastocladiella and female gametangia of Allomyces, on the one hand, and the orange "sporangia" and male gametangia of these two genera, respectively, on the other.

The nuclear apparatus and differentiation.—Cytologically, two important characteristics which differentiate the sex organs in Allomyces remain to be mentioned; the greater number of nuclei per unit volume in the male gametangium, and the larger, basophilic, nuclear cap (again, per unit vol-

ume) in the female gamete.

With respect to the first characteristic, nuclei in fertile, hyphal tips are apparently distributed in random fashion just before septation, but subsequently, the small differentiated male gametangium contains about the same number of nuclei as the much larger (ca. fourfold) female gametangium. It would seem as if the nuclei in male gametangia multiply about four times more rapidly than those in the female following septation (54). Such differential rates of nuclear multiplication in sex organs is certainly not limited to Allomyces, but in most other creatures where this apparently occurs, sex determination is genotypic. On the contrary, in Allomyces sex determination is phenotypic (32). It seems certain that we shall have to start looking for localized cytoplasmic differences (e.g., distributional or architectural variation in Nadi-positive mitochondria, or both, constituents of soluble pools, compounds associated with nucleic acid metabolism discussed below, etc.)

if we are ever to discover and understand the selective mitogenetic mechanisms which lead to differential nuclear multiplication.

As for the second character, the basophilic nature of the nuclear cap in motile cells of Allomyces (and presumably other Blastocladiales; perhaps even Chytridiales) is apparently largely the result of its content of cytoplasmic RNA (67, 68). This may constitute another important clue to the chemistry of sexual differentiation in the Blastocladiales. Basophilic intensity in Allomyces (which appears to be a function of RNA content) is consistenly higher in young, female gametangia than it is in the males; the difference is detectable in the early stage of cytoplasmic granulation, and it becomes more pronounced when nuclear caps are organized around the nuclei at the cleavage stage. But, more important, the increased basophily associated with female gametangia is independent of their position relative to male gametangia; that is, it applies to both epigynous and hypogynous species! This led to the postulation (54) that a potential gradient for RNA synthesis already existed at the disjunction stage, but that it did not become manifested visibly until the maturation stage in gametangial differentiation. Thus, such a gradient in a hypogynous Allomyces would be the inverse of that found in an epigynous species. It seems quite likely that such a gradient in synthetic capacity simply reflects more fundamental gradients in the concentration of mitogenetic substances. The low content of RNA in the cytoplasm of a male organ may be due to the fact that the latter constitutes a zone of intense utilization of RNA, wherein it is converted to the DNA needed for the increased rate of nuclear multiplication found in young male gametangia. Conversely, the more sluggish system of nuclear reproduction in the female would permit greater retention of RNA and, therefore, the larger nuclear caps found in female gametes.

Finally, observations with the electron microscope had revealed numerous pores in the nuclear membranes of Allomyces gametes (69). It was suggested that such pores may have played a role in the exchange of basophilic material during formation of nuclear caps—e.g., its migration from the nucleolus. It is now well known that nuclear caps disintegrate in germinating zoospores or zygotes and that basophilic granular materials then invade the whole cytoplasm. This has been confirmed by recent electron microscopic studies of ultrathin sections of young germlings (70).

Carolenogenesis and differentiation.—The most obvious indicator that metabolic alteration is associated with sexual disjunction in Allomyces is the accumulation of yellow carotenoids, dissolved in the lipide granules of male gametangia. The major component is γ -carotene, the minor one, β -carotene (55). Other polyenes found therein include lycopene and ζ -carotene (58). Traces of the colorless phytofluene were also detected, but it is not known if it was extracted from male gametangia, female gametangia, or mycelium. Gametophytes of A. macrogynous which were rendered colorless with diphenylamine, produced increased amounts of ζ -carotene and phytofluene. In such cultures, the proportion of male sex organs is reduced (75). The rela-

tion between carotenogenesis and androgenesis has been re-emphasized by Foley's (60a) recent studies of albino mutants in Allomyces. In Blastocladiella emersonii, certain correlations have been established between biochemical parameters and the alternate morphogenetic paths leading to genesis of orange and colorless thalli (34, 38, 40). It is tempting to analogize here, and to consider the possibility that carotene synthesis in male gametangia of Allomyces (which, like orange plants of Blastocladiella, are relatively deficient in Nadi-positive mitochondria) may result from a localized interference with normal turnover of the tricarboxylic acid cycle, thus leading to decreased reducing power and an accumulation of carotenoid precursors (e.g., acetate, a carotene building block; see section on Mucorales for references). Indeed, arsenite (an inhibitor of oxidative decarboxylation in the Krebs cycle), and acetate itself promoted genesis of male gametangia in Allomyces (53). Furthermore, the lipide granules of female gametangia seem to possess more "reducing power" than male gametangia, if we accept as suggestive the cytochemical tests with Janus green (phenosafranine) and the plasmalogene reaction (reductive acetal-phosphatides) (71). Once again, although only a beginning has been made, it begins to appear as if the forces which motivate genesis of orange and colorless cells in Allomyces and Blastocladiella may have a common basis.

Environmental control of differentiation: Internal factors influencing epigyny, hypogyny, and the sex ratio.—In the last analysis, it is probably axiomatic that the arrangement of gametangial pairs is ultimately controlled by the genotype; as Emerson & Wilson (72) have shown, a wide range of hybrid forms can be recovered from crosses between A. macrogynus and A. arbuscula. Thus, if biochemical gradients exist (see above), they are probably controlled by a mechanism involving complex polymeric genes (32). In fact, the sex ratio itself also is controlled by genetic factors. For example, interspecific hybrids derived from parents whose male/female ratios were essentially unity, often deviated markedly in this respect; some hybrid gametophytes bore less than one female gametangium for every thousand male gametangia (32)! That such hybrid maleness is associated with some kind of genomic alteration in Allomyces is also evident from the consequences of treating resistant sporangia (for Allomyces meiosporangia; the sites of meiosis) with colchicine or boric acid. Many of the gametophytes derived therefrom exhibit altered male/female ratios (52, 53).

This is an area which is being studied intensively and whose potential bearing on the mechanism of morphogenesis is obvious. At the moment, however, a wide gap still exists between our knowledge of the genetics of the Blastocladiales and the physiology of their differentiation; the interested reader is referred to Emerson (32, 73) for a comprehensive discussion of available information on the former subject.

Environmental control of differentiation: External factors influencing epigyny, hypogyny, and the sex ratio.—Induction of gametangial differentiation in hypotonic solutions was affected by manganese and alcohols (ethanol,

n-butanol) which altered the sex ratio in favor of the females (54); apparently, selective inhibition of differentiation of male gametangia was involved. Diphenylamine, a well-known inhibitor of carotenogenesis (74), also reduces the incidence of male gametangia (75) in at least two species of Allomyces. Conversely, arsenite increased the relative number of male sex organs. Machlis' synthetic medium (10), solidified with agar, was not suitable for illustrating the effects mentioned above because it did not permit vigorous, healthy differentiation of gametangia, especially in A, macrogynus; on this medium, internal lipide degeneration in the gametangia becomes evident early in the development of the gametophyte (53). When acetate was substituted for glucose, the medium became somewhat more favorable for normal differentiation but some female gametangia began to exhibit degeneration of lipide materials. However, incorporation of yeast nucleic acid promoted differentiation of an obviously healthy but predominantly male population of gametangia (53). Substitution of individual nitrogen bases for the nucleic acid revealed that adenine, and more particularly thymine, produced a striking and selective acceleration of male differentiation; with thymine, ratios as high as 95 males/5 females were frequently observed. The most interesting point, however, is that uracil (a pyrimidine base from RNA) does not induce genesis of male gametangia, while thymine (the corresponding base from DNA) had potent androgenic activity. These results may have a direct bearing upon the presumed requirement (see earlier) for increased DNA synthesis during the selective, increased rates of nuclear multiplication in male gametangia (53).

MORPHOGENESIS IN TERRESTRIAL PHYCOMYCETES

In her recent book, Hawker (30) has gathered together and organized into a neat little package, an unwieldy and diversified literature on experimental studies of the reproductive activities of the fungi. What little is known of the fundamental relationship between physiology and morphogenesis in terrestrial Phycomycetes can be found therein; we shall use it as a convenient focal point for the brief consideration of the Mucorales which follows.

Growth in Phycomyces.—Castle's studies on the growth of the sporangiophores of Phycomyces, which have extended over a period of almost two decades, are well known. A digest of his work, as well as references to that of
Blaauw and others who have studied the relationships among wall structures, growth, and sporangiophore development in this fungus, are provided
by Hawker (30) and by Cochrane (87a). The role of indoleacetic acid and
riboflavin in phototropism in Phycomyces has been investigated recently by
Maas (87b), Carlile (87c), and Curry & Gruen (87d). One major piece of
work on this subject which apparently appeared after Hawker's book went
to press but which is summarized and discussed by Cochrane (87a) is that of
Delbruck & Reichardt (76) and, even more recently by Reichardt & Varju
(77). They provide precise information and new insight on both phototropic
responses and light-induced growth responses in sporangiophores of Phyco-

myces, wherein the coupling of illumination, adaptation, and growth are dealt with in detail. The authors discuss some intriguing working hypotheses and, with these, provide a sort of conceptual framework and description for the way in which the responses of *Phycomyces* to stationary and non-stationary illumination may actually operate.

At the moment, little if anything can be said about causal relations between biochemistry and physiology on the one hand, and growth and differentiation of the sporangiophores of *Phycomyces*, on the other. However, at its particular level of integration, the *Phycomyces* story continues to excite the imagination, particularly in the light of these most recent, biophysical investigations.

The relation among environmental factors, sporulation, and sexual reproduction in the Mucorales .- The literature on this subject has been summarized by Hawker (30) and integrated into her coverage of morphogenesis in fungi. Hawker's own extensive work, and the studies by Barnett, Lilly, and numerous others, on the effects of environmental variables such as nutrition, temperature, humidity, carbon dioxide, vitamins, light, the concentration of carbon sources, etc., on sporulation and sexual reproduction, the extensive, early work by Robbins and his co-workers on the role of factors Z1, Z2, and pH in reproduction in Phycomyces, and a good deal of the older literature are included. Actually, not much additional information of this general sort has appeared since that time. Barnett & Lilly (99) have extended their studies of Choanephora, and among other things report that zygospores can be produced in the absence of light and in concentrations of carbon dioxide as high as 10 per cent. Barnett et al. (98) and Hesseltine & Anderson (79) find that paired cultures of opposite mating types of Choanephora produce more carotene than the individual strains alone. On the other hand, Reichel & Wallis (78) were unable to detect such a stimulatory effect with a heterothallic Phycomyces. Zygospore formation in Thamnidium is maximum (80) at rather low temperatures (6° to 7°C.), but the response depends upon the nature of the carbon source (e.g., on certain media, glucose supports zygospore formation while sucrose does not). Hawker et al. (81) have studied the differential effects of temperature upon various stages in the genesis of zygospores in Rhizopus, and are led to the conclusion that a low-temperaturesensitive synthesis of a diffusible substance in the zygospores may be involved. And, finally, a report (82) has appeared on the effect of reduced oxygen tension upon budding in Mucor. Studies of this general kind, wherein the effects of environmental variables upon morphogenesis are established and evaluated, will play an indispensable role in gaining secure footholds from which mechanistic notions of cause-and-effect relationships at a biochemical level of integration will gradually evolve. But, as of the moment, this end has not been achieved as far as the Mucorales are concerned.

One study in particular, however, deserves further mention here—Page's work (83) on the trophocysts and sporangiophores of *Pilobolus*. In this fungus, sporangiophores arise from trophocysts which, in turn, are produced by

the vegetative mycelium. Initiation of trophyocyst formation depends upon a brief exposure of the mycelium to visible light in the 380 to 510 m μ range. The action spectrum contains a broad peak centered around 440 m μ , with a fairly sharp peak at 480 m μ and a possible secondary peak at 410 m μ . Because the action spectrum brought to mind the characteristics of carotenoids and flavines, Page tried to establish which type of pigment might be the more likely light receptor by using inhibitors. He found that the anticarotenogenic agent, diphenylamine, suppressed the formation of orange pigment but did not affect genesis of trophocysts. On the other hand, L-lyxoflavin [reportedly a competitive inhibitor of riboflavin metabolism; see references in Page (83)] not only strongly inhibited trophocysts formation, but its effect was overcome in part by increased exposure of the mycelium to light and almost completely by supplying the mycelium with riboflavin. Thus, Page reasoned, a flavine may have been the light receptor involved in trophocyst formation.

A second aspect of Page's work on this morphogenetic process involved the apparent stimulatory effect of light upon the formation of sporangiophores from the trophocysts, and the fact that this stimulatory effect was accentuated significantly if the trophocysts had previously been subjected to a dark period. These observations, along with others, led Page to suggest that perhaps some chemical substance piles up in trophocysts during the dark period, and that it then sensitizes these structures to light; subsequent exposure of trophocysts to visible light brings about initiation of sporangiophores. Once initiated, such sporangiophores require thiamine or the thiazole portion of the molecule for elongation. It would seem that Page has, indeed, gained a secure foothold upon a problem in morphogenesis which seems beautifully susceptible to further attack on several fronts.

The relationship between metabolism and reproduction in the Mucorales.—Hawker (30) and Cochrane (87a) present a digest of the work of Burgeff, Banbury, and others which has led to the suggestion that certain members of the Mucorales produce diffusible substances ("hormones") which are involved in the initiation and control of zygospore formation. Aside from Plempel's (84) demonstration that sexual reproduction in Mucor seems to involve four stable chemical substances, no other studies have appeared which bear directly upon hormonal phenomena. Perhaps, however, it is an opportune time to ask if the numerous, recent investigations dealing with the physiology and metabolism of these terrestrial Phycomycetes ought to begin to play a vital role, directly or indirectly, in our meditations on past experiments, and in the design of future experiments dealing with the "hormonal" control of zygospore initiation and development in the Mucorales.

In her book, Hawker (30) suggests it is unlikely that fumaric acid or any other relatively stable substance is the active, postulated hormone(s) involved in the experiments of Burgeff and Banbury, because mycelial extracts are devoid of "hormonal" activity. In the absence of additional, definitive information, this is probably the best educated guess that anyone

can make at the moment; the apparent lability of the supposed "hormones" (aside from Plempel's recent work) would appear to be a significant parameter. And yet, it also seems important to remember that both the kinds and amounts of metabolic products liberated by a fungus during growth may bear little resemblance to the quality and quantity of metabolites detectable within homogenates and extracts of that same fungus. Kanie's (85) report that malate, tartrate, and oxalate occur within the cells of *Rhizopus*, while lactate and fumarate are found as external metabolic products would be a case in point for the Mucorales. At any rate, if for no other reason than to focus attention upon the hiatus between metabolism at one extreme and morphogenesis at the other, we have briefly summarized some of the more recent literature of the physiology of the Mucorales in which certain types of metabolic products appear to play a dominant physiological role.

Acetate.—This substance has been reported as a metabolic product of Rhizopus [(86); see also Foster, for earlier references, and Cochrane (87a)]. In Phycomyces, labeled acetate leads to labeled fatty acids during growth on glucose media (88) with the highest specific activity being found in linolenic acid and lesser amounts in C22, C24, and C26 saturated acids. It not only functions as the sole carbon source for growth and carotene synthesis (89), where 50 to 75 per cent of the carotene carbon is then derived from acetate (90), but in fact it seems to induce greater yields of carotene than other substrates such as pyruvate and lactate (91). With Mucor, too, labeled acetate gives rise to labeled carotene (92) wherein acetyl-CoA is considered the probable starting point for carotene synthesis (93) via acetoacetyl CoA (94) and mevalonic acid (95). In Zygorrhynchus, exogenous labeled acetate quickly leads to a large internal pool of labeled amino acids (96). In fact, Moses' (97) observations of the stimulatory effect of acetate (and other Krebs cycle intermediates) upon oxygen consumption in Zygorrhynchus could conceivably be related to Burnett's findings [cf. Hawker (30) for review] that the process leading to copulation in a heterothallic Mucor is associated with increased respiration. And so, if the metabolic machinery for carotene synthesis is related to, and involved in the formation of suspensors, gametangia, and zygotes [(98, 99); other references in Hawker (30); Goodwin (100); etc.], then the formation and metabolism of a potentially volatile (and therefore "labile") substance such as acetic acid might play some causal role in the "hormonal" aspects of zygosporogenesis.

Ethyl alcohol and carbon dioxide.—Alcohol is produced by Rhizopus [(100, 102, 103), and other references in Foster (87)] and by Mucor (104) where thiamine seems to promote its formation from pyruvic acid. Carbon dioxide is reported to stimulate the growth of Rhizopus (105, 106, 107). The possible role of these substances in zygosporogenesis, along with acetaldehyde (108) is, of course, obscure; but, they are highly volatile substances and, as such, could conceivably be involved as "labile hormones."

Pyruvate.—Both Rhizopus (86) and Mucor [Kitahara (104, 109, 110, 111)] may liberate pyruvic acid into the growth medium. In the latter, pyru-

vate accumulation was overcome by providing an adequate supply of thiamine or its pyrimidine moiety, and it was suggested that the internal level of carboxylase activity was the limiting factor under conditions where pyruvate accumulated. Vitamin B2 was also partially effective in reducing the output of pyruvic acid, while all other vitamins tested had no effect. Kitahara believes that vitamin B₁ promotes the conversion of pyruvate to ethyl alcohol while vitamin B2 facilitates oxidation of pyruvate to carbon dioxide and water. And this, of course, leads right back to pathways of caroteniod biosynthesis, and observations such as those of Friend & Goodwin (112) that in Phycomyces, vitamin B1 levels required for maximum growth and those needed for maximum carotene synthesis are not the same. Here again, the established potentiality of some of the Mucorales to liberate pyruvic acid, and the alteration of this potentiality by ingredients of common laboratory media and metabolic products (such as vitamins), deserve consideration in attempts to understand and to design experiments dealing with the physiology of copulation and genesis of zygospores.

Lactate and fumarate.—These compounds are well-established products of the growth and metabolism of Rhizopus [(101 to 104, 109, 110, 113, 114 to 118), and references in Foster (87) and Cochrane (87a)]. Insofar as they are relatively stable chemically and apparently end products of metabolism in most instances, probably they are not [as Hawker (30) has suggested] very suspectable potential "hormones." Nonetheless, it would be good to have available some direct evidence which rules them out of the picture.

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ANTIBIOTICS IN FOOD PRESERVATION1

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INTRODUCTION

The history of the development of food preservation methods may be divided into a number of stages or eras, depending upon the techniques used to maintain the foods in an edible state. The first era, dating back to prehistoric and extending into modern times, comprises such processes as sun drying, smoking, salting, pickling or marinating with acids and spices and combinations of these, certain types of fermentations, and artificial dehydration. The second stage, foreshadowed by Papin's invention of his digester or pressure cooker in 1681, had its inception in 1810 with the heating of food in closed containers by Appert. This led to the establishment of the canning industry in the early 19th century in Europe and in the late 19th century in the United States where, in the first half of the 20th century, it has become the leading food preservation industry. However, food preserved by canning is generally changed from its original state. Furthermore, many foods do not lend themselves very readily to the canning operation or would not be looked upon favorably by the consuming public if they were to be canned. The third stage may be called the refrigeration era, which received its main impetus with the development of mechanical refrigeration equipment in the last quarter of the 19th century. This means of preserving food expanded slowly and has become of commercial importance and stature only within the second quarter of the 20th century. Even though refrigeration without freezing offers the best means of maintaining most perishable foodstuffs in a condition as close as possible to their original state, nevertheless such perishables have only a rather limited storage life. The generally recognized perishability under optimum storage conditions of such foods as fish, meat, milk, eggs, and vegetables is evidence of the limitation of the refrigeration process. Cutting (1) has written a very thorough review of the methods comprising the first three stages, particularly in relation to fish. The fourth stage is one which is still in its very early infancy or even fetal stage. This comprises the combined use of antibiotics and refrigeration to extend appreciably the edibility of perishable foodstuffs. A fifth stage may be foreseen in the future, in which the combination of antibiotics with irradiation and refrigeration could protect the perishable foodstuffs to a still greater extent beyond the limits presently possible with antibiotics and refrigeration. Sufficient preliminary and exploratory experiments have been carried out to make the prospects for the successful application of this triple combination very favorable.

¹ The survey of the literature pertaining to this review was concluded in January, 1959.

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The first of the modern antibiotics, penicillin, was discovered in 1929 by Fleming (2). However, the "era of antibiotics" in medicine and therapeutics may be considered to have actually begun in 1941 with the clinical use of penicillin (3). Similarly, the "antibiotic era" of food preservation may be considered to have had its potential inception in 1948 with the discovery by Duggar (4) of Aureomycin or chlortetracycline. The commercial application of an antibiotic for perishable food preservation became possible on November 30, 1955, when the Food and Drug Administration cleared the use of chlortetracycline and established a tolerance of 7 p.p.m. for residues of chlortetracycline in or on uncooked poultry. The Dominion of Canada cleared the use of this substance for the preservation of poultry and fish on September 26, 1956 and set tolerances of 7 and 5 p.p.m., respectively, in the uncooked products. Later, the United States and Canada extended their approval for the similar use of oxytetracycline with the same tolerances.

Antibiotics as Food Preservatives

A number of reviews of various aspects of the subject have been published (5 to 16). The present review represents a survey of the applications of antibiotics for the preservation of perishable foodstuffs and food products from their introduction in the early 1940's to the end of 1958, together with some discussion of their possible public health implications and hazards, and an examination of their possible role in food technology. To simplify the presentation the various categories of perishable foods are discussed separately.

Fish.—The ineffectiveness of such narrow-spectrum antibiotics as penicillic acid, penicillin, and streptomycin for the preservation of fish, compared to the activity of sulfa drugs and sodium nitrite, was found out soon after their introduction (17, 18). Following the discovery of the so-called broadspectrum antibiotics, a number of reports appeared which indicated their potential usefulness for fish preservation. Tarr et al. showed that chlortetracycline, oxytetracycline, and chloramphenicol at 10 to 25 p.p.m. inhibited bacterial growth in minced halibut, salmon, and brill up to 10 days at 33° and 37°C. (19). They also examined the activities of 14 antibiotics and found that these same three antibiotics were the most effective in controlling the growth of the mixed flora of fish at 0° to 21°C. (20). Spoilage of whole eviscerated fish was markedly retarded by ices containing 1 to 4 p.p.m. of chlortetracycline, by holding in sea water containing 2 p.p.m. at -1°C. for six days, and by a 1-min. dip in a 50 or 100 p.p.m. chlortetracycline solution prior to icing (21). The effectiveness of this antibiotic in ice or in seawater solution has been shown in a number of reports by Tarr and his co-workers for various fish species, including salmon, halibut, lingcod, gray cod, flatfish, and sablefish or black cod (22 to 26). Tarr has repeatedly stated in these various reports that, of a large number of antibiotic preparations including nitrofurans, the three tetracyclines, chlor-, oxy-, and tetracycline, and particularly the first, CTC, were the most effective agents for the preservation of fish (27).

In a series of papers from the laboratories of the American Cyanamid Company it has been reported that spoilage of such fish as sea bass, weakfish, croaker, butterfish, porgy, scrod, salmon, halibut, and redfish has been delayed and the freshness appreciably extended by chlortetracycline dips in 5 to 25 p.p.m. solutions in seawater, by storage in ice containing 5 p.p.m., and in refrigerated brine containing 5 and 10 p.p.m. (28, 29, 30). Stern et al. have reported that at a concentration of 2 to 5 p.p.m. in 3 per cent salt solutions chlor-, oxy-, and tetracycline were about equally effective in extending the keeping quality of English sole beyond that accomplished in plain brine at 0 to 2°C. (31). These workers have also reported that the three tetracyclines at levels of 5, 10, and 20 p.p.m. in salt brines at 32°, 40°, and 50°F. were equally effective in preventing spoilage of round salmon compared to storage in plain brine, and that their effectiveness decreased with increasing storage temperature. No significant differences were noted between the effects of the various concentrations of the antibiotics (32, 33).

In a preliminary study by the writer it was found that at a concentration of 2 p.p.m. in 5 per cent salt solutions, neomycin was ineffective, whereas chlortetracycline and oxytetracycline were effective in prolonging the storage life of lingcod and sablefish fillets at 2.8 to 9.4°C., the former antibiotic being somewhat more active than the latter [Farber (34)]. A study of the effect of a 2-min. dip in 5 and 25 p.p.m. in 5 per cent solutions of 14 antibiotics, including penicillin, bacitracin, carbomycin (Magnamycin), polymyxin B, rimocidin, tyrothricin, neomycin, streptomycin, cycloserine, amimycin, chloramphenical, tetracycline, oxytetracycline and chlortetracycline, on the rate of spoilage of sole fillets at 5.5°C. (42°F.) was next carried out. The first 10 preparations had no significant preservative action on the fillets, as shown by chemical, bacteriological, and sensory tests. The last four antibiotics slowed the rate of spoilage, chlortetracycline being the most active. Furthermore, of these four antibiotics only chlortetracycline was equally effective at 5 and 25 p.p.m., whereas the other three were somewhat more effective at the higher concentration (35). Ineffectiveness of four other antibiotics, and the superiority of chlortetracycline particularly at lower temperatures was reported by Lerke & Farber (36). Some plant-scale, semi-commercial tests were made on round flatfish and on fillets of flatfish and of rockfish, with ice containing 5 p.p.m. and dips containing 5 and 10 p.p.m. of chlortetracycline, respectively. The antibiotic treatments markedly prolonged the time during which the fish remained fresh, in contrast to control fish in ordinary ice and those which were dipped in 5 per cent salt solution (37, 38). The ineffectiveness as preservatives of sole fillets of thiostrepton, albamycin, amphotericin A and B, nystatin (Mycostatin), ristocetin, vancomycin, oleandomycin, erythromycin, and such furan derivatives as nitrofurazone (Furacin), and microfur was shown by Lerke in this laboratory (39). He also showed that novobiocin, furoxone, and nitrofurantoin (Furadantin) had a slight effect by delaying the spoilage of sole fillets at 5°C. for one to two days. Mixtures of chlortetracycline with streptomycin, with neomycin, and with nitrofu128 FARBER

rantoin were no more effective than when used alone; as was the mixture of tetracycline with oleandomycin. The combination of chlortetracycline with novobiocin delayed the spoilage of sole fillets at 5°C. two to three days longer than did chlortetracycline alone [Lerke (39)].

Tomiyama et al. have also reported on the prolongation of freshness of various species of round fish by ice containing 5 p.p.m. of chlortetracycline with and without a previous immersion in a 10 p.p.m. chlortetracycline solution. Sardines, mackerel, herring, croaker, sea bream, and sole have been kept for 8 to 13 days and have been transported 300 miles after landing in a good condition (40 to 44).

Shewan, at the Torry Research Station, Scotland, has confirmed the beneficial effect of chlortetracycline ice on the storage life of gutted and washed white fish on board a trawler and on shore (45, 46, 47). Haddock, cod, whiting, and lemon sole in ice containing 5 p.p.m. chlortetracycline organoleptically were still judged edible after 21 days, whereas in ordinary ice they were just passable after 14 days. Fillets which were dipped in a 20 p.p.m. aqueous solution for 5 min. remained fresh longer at 5° and 15°C. than control fillets

dipped in water.

Similar results confirming the efficacy of CTC in delaying spoilage of fish have been reported from various other countries [Hjorth-Hansen, Norway (50); Meyersberg, Austria (51); Sebastio, Italy (53); Ducroz, French Cameroons (54)]. In contrast to these investigators, Bystedt & Liljemark in Sweden reported that ice containing 5 p.p.m. chlortetracycline inhibited bacterial growth in gutted haddock during five days on a fishing boat and during subsequent storage in ice ashore, up to a total storage period of 14 days. However, no effect was observed on the increase in pH and in trimethylamine content and on the decrease in organoleptic score, compared with the fish in plain ice (49). Albertsen in Denmark carried out some storage tests on cod and plaice in plain ice and in ice containing 2 to 5 p.p.m. chlortetracycline at 8°C. (46.4°F.). He reported a three- to four-day extension of the storage time in the chlortetracycline ice beyond that in the plain ice (48). However, he stated that "our experiments with chlortetracycline ice have not shown nearly as prolonged a keeping quality of cod and plaice as the investigations carried out at the Torry Research Station and by Tarr in Canada." A possible explanation for this observation may be that the fish at the start of his experiments had already lost part of their storage life, since their trimethylamine content was given as 10 to 12 mg, of nitrogen per 100 gm. fish, a value generally considered to be found in fish past their original freshness or even in the first stages of deterioration. Castell (52) has reported that haddock fillets, which had been given a 10 p.p.m. chlorotetracycline dip, kept about four days longer in aluminum containers buried in ice at 32°F. than when they were tightly wrapped. The trimethylamine values for untreated unwrapped, untreated wrapped, treated unwrapped, and treated wrapped fillets before and after storage were 0.15 to 2.97, 0.18 to 15.64, 0.18 to 0.65, and 0.20 to 2.04 mg. nitrogen per 100 gm. of fish, respectively.

The general observation that can be made from the studies on fish preservation by antibiotics is the almost unanimous finding that the tetracyclines, and particularly chlortetracycline which has been tested to the greatest extent, will appreciably prolong the storage life and delay spoilage of fish and fillets, the actual increase in keeping time depending among other factors on the storage temperature and on the freshness of the starting material. A number of reviews on the effects of antibiotics on fish preservation have appeared (24, 55, 56, 57).

Shellfish.—The preservative effect of antibiotics on shellfish is somewhat complicated by the fact that some shellfish are handled in both a raw and a cooked state. Furthermore, the presence of the shell or "peel" on shrimp adds another complicating factor. As a result, the literature on the efficacy of antibiotics in prolonging the freshness of shellfish contains more discrepancies and conflicting results than that for fish. Nevertheless, there are a number of reports which are unequivocal and show the potential beneficial effect of some antibiotics for shellfish.

In one of the early studies on the effect of antibiotics on shrimp freshness, Farber found that immersion of raw beheaded unpeeled shrimp in a 2 p.p.m. chlortetracycline in 5 per cent salt solution did not retard spoilage (34). Later studies from this laboratory have shown that a 5-min. dip in a 15 p.p.m. solution definitely prolonged the freshness of both peeled and unpeeled raw beheaded shrimp and that the actual extension of the keeping quality depended upon the storage temperature. Concentrations greater than 15 to 25 p.p.m. were not significantly more effective on the shrimp-keeping quality. Peeled raw shrimp were benefited to a much greater extent by the 15 p.p.m. chlortetracycline treatment than were unpeeled raw shrimp (36, 39, 61). Higman et al. (59) showed that the undesirable odors which developed in shrimp held in refrigerated sea water (58) could be eliminated by the addition of 5 to 10 p.p.m. chlortetracycline to the refrigerated storage medium. From the same laboratory it was reported by Camber (60) that 10 to 100 p.p.m. chlortetracycline dips and 10 p.p.m. in ice were effective in prolonging the storage life of shrimp. Fieger et al. found that ice containing 10 p.p.m. extended the storage life of shrimp by two to four days, but did not have any controlling action on black spot formation or melanosis (62). Novak et al. reported that a 20 p.p.m. chlortetracycline dip preserved oysters prior to their storage in ice or by freezing (63). Boyd & Tarr reported that immersion of shucked oysters for 3 min. in 20 p.p.m. chlortetracycline solution had a delaying action on their bacterial spoilage, lower concentrations being ineffective (64). On the other hand, Abbey et al. stated that a wash in a 5 to 10 p.p.m. solution reduced the bacterial counts of shucked oysters and appreciably extended their storage life compared to the control oysters (65). Benarde & Littleford (66) reported that even though chlor- and oxytetracycline treatments decreased the bacterial counts of crabmeat and oysters they did not extend their shelf life beyond that of the untreated controls, as judged organoleptically. Benarde also reported that 10 p.p.m. solutions of chlortetracy130 FARBER

cline, oxytetracycline, oleandomycin, polymyxin B and a mixture of oxytetracycline and polymyxin B did not prolong the storage life of cooked crabmeat stored at 1 to 3°C. (67). Tarr et al. (20) reported that both chlor- and oxytetracycline at 20 p.p.m. prolonged the storage life of cooked crabmeat for a few days.

Poultry.—A number of investigators in this and other countries have reported that the addition of 10 p.p.m. chlortetracycline to the chill tanks resulted in prolonging the shelf life of whole eviscerated or cut-up chickens and of turkeys by 3 to 14 or more days, depending upon the storage temperature (68 to 73, 75, 76, 81, 82, 83). The superiority of chlortetracycline over other antibiotics for prolonging the storage life of poultry has been broughtout in various studies (74, 78, 80, 84). Shrimpton et al. found that when chloroxy-, and tetracycline, or a mixture of bacitracin and neomycin were given in the drinking water within 24 hr. before slaughter the resulting carcasses had a longer shelf life than the controls receiving only water (77). In contrast to the above studies which showed the superiority of antibiotic-treated poultry, as judged in various ways, Baker (79) reported that taste panel scores for fresh flavor were higher for untreated than for chlortetracycline-treated poultry. Shrimpton (76) and Yacowitz et al. (82) have also pointed out that yeasts and molds become prominent in antibiotic-treated poultry, and the latter have also shown that a mixture of 10 p.p.m. chlortetracycline with 5 to 10 p.p.m. of nystatin prevented the growth of yeasts and molds and the development of resulting spoilage odors on chicken parts during storage.

Meat and meat products.—A series of papers on the application of antibiotics for meat preservation have been published by the Ohio State University group of Deatherage, Weiser and co-workers. Of six antibiotics tested in ground beef, only chlortetracycline, oxytetracycline, and chloramphenicol at 0.5 to 2 p.p.m. extended the storage life beyond that of the untreated controls and inhibited the growth of a number of organisms isolated from spoiled meat (85). Rounds and whole animals were infused with chlortetracycline to put about 2 p.p.m. into the meat. The storage life of the treated rounds and of the cut-up meat from the infused animals at room temperature and in a chill room was prolonged beyond that of untreated meat. The tenderization of steaks could be speeded up by a 48-hr. room temperature storage followed by a five-day chilling to equal that found at two weeks postmortem in untreated controls (86). Similar results for the preservative effect of 2 p.p.m. chlortetracycline and chloramphenicol on ground beef and on the microflora present were reported later (87). The unreliability of in vitro tests on spoilage organisms to evaluate the potential effectiveness of antibiotics has been discussed in a number of papers by Jay, Weiser & Deatherage (88, 89, 90). They showed that 5 p.p.m. chlortetracycline would prevent spoilage in beef which had been inoculated with a culture of an organism which was reistant to 50 p.p.m. A discussion of the use of antibiotics in meat preservation has been presented by Cahill (91). Tarr et al. have also reported that the storage life of ground beef, beef shank meat, and of round steaks was extended by 1 to 10 p.p.m. chlor- and oxytetracycline, with the former being somewhat more effective (20, 21). The yeasts which tended to predominate as storage progressed were markedly reduced by the addition of 10 to 20 p.p.m. rimocidin to the chlortetracycline (20). The beneficial effect on the subsequent storage life of the meat of an intraperitoneal injection of oxytetracycline into lambs, cattle, sheep, and hogs one to two hours before slaughter was reported by a group from the Pfizer laboratories (92, 93, 94, 96), Levels of 1 to 6 mg, per pound of body weight were used. Spoilage of the carcasses was inhibited for a few days even at 80 to 100°F. Spraying of the open surfaces of the carcases with a mixture containing 0.5 per cent carboxymethyl cellulose, 10 p.p.m. oxytetracycline, and a wetting agent improved the appearance of the meat (95). The preservative effect of 10 p.p.m. chlortetracycline dips on meat stored at 2 to 3°C. has been reported in Russia (96). It has been reported in Poland that the injection of 25,000 I.U. of penicillin per kg. of body weights into rabbits and calves 20 min, prior to slaughter resulted in a three- to fourday increase in storage life of the meat at 16 to 20°C, beyond that of the untreated controls (97). Ordal & Brown (98) have reported results on ground lean pork treated with oxytetracycline and salt. Oxytetracycline at 10 p.p.m. extended the storage time for about four days, and for about 10 days in combination with 3 per cent salt. Curing solutions for hams containing 15 to 45 p.p.m. oxytetracycline and 4 per cent salt gave hams a longer storage life at 37°F. A dip in a 1 per cent chloramphenical solution was less effective in preventing the growth of enterotoxigenic Staphylococci or Salmonellae on the surface of beef and pork than a 1 per cent acetic acid solution (99).

Canned foods.-The report by Andersen & Michener (100) that 5 to 20 p.p.m. of subtilin in conjunction with a 5-to-10 min, heating at 212°F, prevented food spoilage and the growth of toxic clostridia started an extensive series of studies on the possible use of subtilin and other antibiotics in canning. The goal was the reduction of the rather long heat treatments necessary to sterilize low acid foods safely and to prevent thermophilic spoilage. Even though subtilin with a mild heat treatment was found to have a sporostatic action on spores of Clostridium botulinum and of thermophilic flat-sour organisms (103), and to lower their thermal resistance (108, 109), others reported that at most the spoilage was only delayed by subtilin and other antibiotics (101, 102, 105, 107, 108, 111, 112). Michener (104) reported a variation in the susceptibility of a thermophilic flat-sour organism to subtilin arising from the presence of smooth and rough variants with differing sensitivities. The development of resistance of various microorganisms to subtilin has been reported (113, 114). Spilde (115) reported that canned fish balls spoiled in the presence of subtilin after 15 min. at 100°C. It is generally accepted that subtilin, nisin, and other antibiotics which have been reported to be active against sporeformers are, at most, sporostatic and that they probably act when the spores germinate to vegetative cells (103, 104, 116). Subtilin and nisin along with mild heating have been found to inhibit flat-sour thermophilic spoilage of tomato juice (105 to 108, 121). Williams et 132 FARBER

al. have reported the existence of strains of C. botulinum and of thermophiles with varying resistance to subtilin and that the resistance increased with increasing numbers of spores in the medium (117, 118). Wynne et al. have found that neither penicillin nor streptomycin inhibited the germination of food poisoning and food-spoiling clostridia (119, 120). Reviews of the possible use of antibiotics in canning, and discussions of the problems involved and of the safety factors necessary to be determined before their practical use in canning have been published (122 to 125). The situation has been quite well summed up by Hawley (121) who stated that antibiotics should not be used for purposes of reducing heat treatment of canned foods which must be rendered safe against Clostridium botulinum. Nisin (and subtilin) may be used to supplement normal processing schedules as a means of controlling spoilage by organisms resisting normal heat treatment (such as thermophiles).

Dairy products.—The findings of Curran & Evans (126) that penicillin in milk was sporocidal against aerobic sporeformers and flat-sour spoilage organisms, and of Foley & Byrnes (130) that penicillin inhibited the natural milk flora, were later shown not to be of practical use, since neither penicillin, nor streptomycin, nor subtilin with a mild heating prevented the spoilage of normal and concentrated milk (127, 128, 129). For short-term preservation chlortetracycline (131) and streptomycin (132, 133) were found effective for human milk. Cow's milk was preserved for periods to 8 to 10 days at 30°C. by mixtures of 100 p.p.m. chlortetracycline with 100 p.p.m. of patulin and penicillin, and for three days by 20 p.p.m. of these antibiotics (134, 136). Raw cow's milk was preserved for one, two, three, and four days at 30°C. by 200 p.p.m. penicillin and streptomycin, 100 p.p.m. chloramphenicol, 200 p.p.m. patulin, and 100 p.p.m. chlortetracycline and oxytetracycline, respectively. Pasteurized milk was preserved for 12 to 15 days at 30°C, by the same antibiotics (135). Shahani et al. also found that pasteurized milk was protected longer than raw cow's milk by penicillin, streptomycin, chlor- and oxytetracycline at 25 p.p.m. (137). Shiveler et al. (138) reported that raw milk was preserved for one day by 3 p.p.m. of penicillin, 10 p.p.m. each of dehydrostreptomycin and of chlortetacycline, whereas pasteurized milk kept for 48 hr.; chlortetracycline was the most effective of the preparations tried. Similar results on increasing the storage life of milk by chlortetracycline and oxytetracycline were reported by Angelotti et al. (139) and by Green & Bell (143), Nisin, the antibiotic produced by certain strains of Streptococcus lactis (140), has been reported to control clostridial spoilage in cheese (141, 142).

Fruits and vegetables.—Smith and Brody & Francis have reported that the shelf life of packaged spinach which has been inoculated with soft-rot-producing bacteria was extended by a dip or spray of 0.5 to 0.1 per cent streptomycin or oxytetracycline for one day at 21.1°C. (144, 146, 147). Streptomycin at 0.1 per cent concentration also controlled bacterial soft-rot of packaged cole slaw for 3 days (145). Potato slices were protected from black-leg rot by streptomycin and dihydrostreptomycin and from Pseudomonas

fluorescens rot by chlortetracycline (148). Bacterial spoilage of vegetables was delayed up to 48 hr. by 25 and 50 p.p.m. oxytetracycline (149). Salad vegetables, singly and in mixtures, were preserved for 1, 7, and 12 days at 30, 10, and 5°C., respectively, by 25 to 50 p.p.m. oxytetracycline (150). Strawberries were protected from mixed Rhizopus stolonifer and Botrytiscinerea rot for 48 to 72 hr. at 21°C. by 100 p.p.m. dips in the antifungal antibiotics cycloheximide and oligomycin (153). Nagel & Vaughn showed that the combination of heating at 71°C. with 1000 units of penicillin and 200 µg. streptomycin per ml. of brine sterilized cucumbers with no significant texture changes (151). Fresh grapes were preserved at 2°C. for 2 months beyond controls by a penicillin dip (152).

Miscellaneous preparations—Godkin & Cathcart (154) reported that 0.6 and 1 p.p.m. chlor- and oxytetracycline prevented the growth of Micrococcus pyogenes in custard fillings for 24 hr. at 37°C., but not that of the normal heat-resistant flora. The same authors later showed that a mixture of 10 p.p.m. oxytetracycline with 70 p.p.m. subtilin retarded the growth of food poisoning enterococci, salmonellae, and micrococci and of the normal heat resistant spoilage organisms found in custard filling for three days during summer weather (155). The saké-putrefying bacteria have been inhibited by 20 p.p.m. of chlor- and oxytetracycline and by more than 10 p.p.m. subtilin (156, 157). The Gram-negative contaminating bacteria in brewer's yeast were found to be effectively controlled by 0.0005 to 0.05 units per ml. of polymyxin B (158, 159). The Gram-positive lactic bacteria in finished beer were most effectively controlled by penicillin (158).

PUBLIC HEALTH ASPECTS

A number of factors of possible public health significance have to be taken into account before the use of antibiotics for perishable food preservation can become widespread. Among these are oral toxicity, the development of sensitivity which may influence later therapeutic use, the development of a resistant flora which may produce an enhanced spoilage, and the development of a resistant pathogenic flora which could affect later therapy. Thatcher (160) has reviewed some of the public health aspects of antibiotics in foods and has discussed the above problems. The tetracycline group of antibiotics is characterized by a step-by-step or progressive type of resistance development by bacteria, in contrast to what may be called the "one-step" development of a high resistance to such agents as streptomycin. Since "the microbial contaminants on each incoming consignment of untreated foods will normally have had no previous contact with the antibiotic, repetitive exposure to a particular antibiotic will not occur, and, in the instance of the tetracyclines, a high degree of resistance would not be expected to develop," and "a single exposure in foods, at the low levels recommended, to a member of the tetracycline group . . . is unlikely to induce resistance to the much higher concentrations therapeutically used" (160). Finland (161) reported that the large-scale use of antibiotics in the feed of meat animals has not led

to the emergence of a numerically dominant population of resistant pathogens. From a review of the effects of the long-term administration of chlortetracycline to man and animals, Hines (162) concluded that "there is no evidence to indicate that the prolonged oral administration of chlortetracycline to man or domestic animals is harmful at presently recommended dose levels." Levels recommended for application to human food are much lower than the dosage levels for feeds or therapy. "Hence it seems reasonable to accept the thesis that tetracyclines applied to foods at the recommended rates would not be toxic per se" (162). In an editorial (163), it was stated that physicians who had given geriatric patients chlortetracycline daily for periods of 20 months to 4 years found no significant indication of the build-up of resistant strains or of sensitization reactions in those receiving it in contrast to those receiving placebos. Thatcher (160) concluded from his review of the literature that "such inquiry as has been possible indicates that for practical purposes the tetracyclines should be considered non-allergenic, since bona fide cases are extremely rare even among production workers or those who are in constant contact with the tetracyclines used in animal feed."

Some of the antibiotics have obvious disadvantages which may preclude their widespread practical application. For example, streptomycin is fairly heat resistant and active residues are found in treated vegetables even after cooking (164). This fact, together with the "one-step," high-level resistance which streptomycin tends to produce and the hypersensitivity to it which may develop (166), are possible reasons against its general use in foods. With the usual levels of chlor- and oxytetracycline which have been used in fish, the resulting residues in the flesh of the raw product have been in the range of 1 to 2 p.p.m. (37, 39, 169, 170). The skin of such fish as tuna, sardines, and mackerel has been found to prevent penetration of chlor- and oxytetracycline into the deeper layers of flesh and canning has been shown to inactivate these antibiotics completely (32, 33, 39). The above facts, together with the observation that chlortetracycline is likely inactivated in the gastrointestinal tract (171), are arguments in favor of the preferred use of the tetracycline antibiotics in food preservation.

PRACTICAL CONSIDERATIONS

Kersey et al. have stated that it appears to be unlikely that antibiotics alone could ever afford a practical means of food sterilization. Wrenshall (15) wrote "it is not contemplated that the use of antibiotics will displace canning, freezing, dehydration, curing, or any conventional method of food preservation. The use of antibiotics can supplement and improve such processes by preventing bacterial deterioration before the final act of preservation can be accomplished." Durbin (165) has stated that "neither refrigeration nor antibiotics alone will sterilize food. Antibiotics alone may add sufficient time to the keeping quality of some foods to permit more efficient and less wasteful distribution of the food. Since antibiotics may only inhibit bacterial growth and not destroy it directly they are useful where the total number of

potential spoilage organisms is low." Therefore, it is important that the producer of a product on which antibiotics are to be used be doubly careful in preventing contamination of the food products during processing. Kline (167) wrote that "It should be kept in mind that Acronize (a food-grade chlortetracycline preparation) must never be used as a substitute for sanitation or refrigeration, nor as a redemption measure for poor or marginal fish products. Rather it has been designed to enhance the value of good sanitary and refrigeration practices." Similar ideas have been expressed by Deatherage (8) who said "refrigeration and antibiotics are complementary since both can do more than either alone in controlling spoilage. Both are most effective in controlling spoilage when food is handled in the most sanitary manner. Neither can be used to upgrade a spoiled or inferior product. Antibiotics are most effective in the most sanitary and cleanest operations." The importance of the interaction of the three factors, antibiotics, refrigeration, and sanitation will always have to be borne in mind by those using antibiotics to preserve perishable foodstuffs. This also has been emphasized by Miller (168). The potential preservative value of an antibiotic such as chlortetracycline can be partially or completely vitiated by a lack of either refrigeration or proper sanitation. This has been pointed out among others by Vaughn et al. (80) and by Wells et al. (74) for poultry applications and by Lerke (39) for fish and shellfish.

With the proper use of such effective antibiotics as chlor- or oxytetracycline in conjunction with low temperatures near 0°C. and sanitary operating conditions, major developments can be expected, for example, in the more widespread distribution and marketing of fish and shellfish (38), in the distribution and consumption of meat, milk, and other perishables in tropical countries where adequate refrigeration facilities are not available, and in raising the nutritive level of the diets of many underdeveloped countries, by enabling such perishable foods as fresh milk, meat, and fish to be distributed to areas where these foods are now unavailable. Given the proper circumstances a new era in perishable food preservation can truly be ahead.

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MICROBIOLOGICAL ASPECTS OF TISSUE CULTURE^{1,2}

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During the first half century which has elapsed since the classic experiments of Harrison, tissue culture has played an ever-increasing role in practically every field of experimental biology as witnessed by the vast literature on the subject (1). The term "tissue culture" with reference to mammalian species can be divided into two broad categories. One involves the study of organized groups of cells from the standpoint of functional and anatomical development. This important segment is germane to experimental embryology and related areas of physiology and it is beyond the scope of this review. A second aspect of tissue culture, which provides the basis for this discussion, is concerned with the survival or proliferation of cells derived from various tissues. In this connection, the term "tissue culture" is in itself a misnomer since it is the constituent cells of the tissues that are grown, rather than the tissues themselves, as structural entities. For purposes of the present discussion and to conform with much of the literature, the terms "cell culture" and "tissue culture" will be used interchangeably.

During the last decade, cell culture has evolved to a point where its status is remarkably similar to that of bacteriology two generations ago. Methods have been developed which permit the serial propagation of many strains of mammalian cells with the same facility as that of the more fastidious and slow-growing microorganisms. Sufficient information on the nutrition and physiology of mammalian cells *in vitro* has been gained to permit embryonic development of studies in certain areas of intermediary metabolism and genetics.

This review is intended to record some of those developments which have ushered in the "microbiological era" of cell culture. To accomplish this it has been neessary to be somewhat arbitrary in the selection and presentation of the material. The year 1948 has been selected as the point of departure of "modern" cell culture from the earlier methods. Traditionally, studies of cell culture have been so intimately integrated with the applied aspects of the tissue culture system that it becomes difficult to delineate the boundaries of the subject in relation to the study of cells per se. Examples of the appli-

¹ The survey of the literature pertaining to this review was concluded in December, 1958.

² The following abbreviations will be used: DNA (deoxyribonucleic acid); DNAP (deoxyribonucleic acid phosphorus); DPN (diphosphopyridine nucleotide); RNA (ribonucleic acid).

^a The author is indebted to Drs. R. F. Parker and L. O. Krampitz for many helpful suggestions concerning the preparation of this manuscript.

cation of tissue culture to virology can be found in recent reviews by Ross & Syverton (2), Enders (3, 4), Dulbecco (5), and Walker et al. (6). Similarly, the contribution of cell culture to cancer research has been discussed recently by Leighton (7) and by Hirschberg (8). Neither of these topics will be considered except in instances in which they are related directly to problems in cell culture. The limitations imposed by space have made it impossible to include more than half of the papers which have appeared on this subject. Accordingly, it has been necessary to review the literature briefly and, in some instances, cursorily. Thus, the reader may find the presentation more provocative than informative.

CELL CULTURE METHODOLOGY

Elimination of the plasma coagulum.—The early methods were based on the observation that cells from many tissues would proliferate in a plasma coagulum when nourished with media containing materials of animal origin such as serum, ascitic fluid, and tissue extracts. The methods evolved relied on aseptic techniques and made use of cover slips, specially designed flasks, and rotating tubes. These methods are described in detail in several recent texts (9 to 13). Plasma was considered to serve as source of nutrients and as a matrix for the support of cells, which was believed to be an indispensable physical requirement. The limitations and disadvantages of the plasma system have been pointed out repeatedly (9, 14, 15, 16). As the culmination of a long series of experiments, Earle and his co-workers (17, 18, 19) demonstrated that a variety of cells would proliferate under, and in contact with, a sheet of perforated cellophane in a medium containing serum and embryo extract. It was also observed that cells could be scraped from the cellophane, suspended in fluid medium and planted as a cell suspension in a new culture (20). It was found later that the cellophane could be omitted entirely. The cells settled out directly on the glass floor of the flask and proliferated there as luxuriantly as they had on cellophane (21, 22). That cells would proliferate on glass in the absence of plasma was not an entirely new concept (14, 23, 24) in 1950, but in a manner analogous to Harrison's original experiments the studies made by Earle and his colleagues demonstrated the simplicity and practical aspects of the system. In the opinion of the author, the experiments demonstrating the feasibility of growing cells on glass in the absence of a plasma coagulum represented the greatest single advance in cell culture in the preceding thirty years, by virtue of the fact that it laid the foundation for the practical application of the microbiological procedures to the cultivation of mammalian cells.

Dispersal of cells.—The preparation of uniform suspensions of cells is an important prerequisite not only for the successful propagation of cells on glass, but also for their use in quantitative experiments. Scherer et al. (25) revived the Rous-Jones procedure of trypsin digestion and used it to disperse cells grown on glass. Various modifications (26, 27) of the trypsin procedure are now used routinely in the preparation of suspensions from cell

cultures. Frisch & Jentoft (28) used trypsin to prepare primary cultures of monkey testicle. This procedure has been refined (29 to 32) and applied to other tissues. Trypsin is not entirely innocuous and under certain conditions may damage cells (33, 34). Puck et al. (35) have stressed the importance of shortening the exposure time and minimizing mechanical trauma during trypsinization. The separation of cells by the chelating action of versene introduced by Zwilling (36) has not been used extensively. Mechanical procedures may yield adequate suspensions when the constituent cells of the culture separate after division (21, 22, 37).

Quantitative aspects of cell culture.- To pursue quantitative studies with cells, it is essential to have a rapid and reliable method for enumerating them. The direct methods which are suitable include the determination of packed cell volume (38), enumeration of trypsin-dispersed cells (39), or nuclei released from cells by treatment with citric acid (40). The nuclear counting procedure introduced by Sanford et al. (40), has a number of advantages in that it circumvents problems of cell clumping and can be applied to cells in situ. Parker et al. (41) have modified the procedure considerably and simplified versions of the method have been used in a number of laboratories (42, 43, 44). The earlier chemical methods (9, 45) are based on the rate of metabolic production or utilization of some compound which can be readily quantitated. Such methods are limited to use under carefully circumscribed conditions because the rate at which substrates are metabolized is a function of the physiological state of the cells or their environment, or both. Attention has been directed recently to the determination of various cellular components as a measure of proliferation. Healy et al. employed DNAP (46), McIntire & Sproull (47) described a simplified method for measuring DNA, Oyama & Eagle (48) adapted the Lowry protein method for use in cell culture. McIntire & Smith (49) determined total purines and pyrimidines. The latter two methods are commendable in their simplicity but it should be noted that the quantity of protein and total purines and pyrimidines per cell vary with the conditions of growth (48, 49). The methods of Evans et al. (37) and Parker et al. (41) are prototypes for the preparation of replicate cultures en masse. These have been modified to satisfy the needs of individual laboratories (29, 39, 42, 44).

Cloning techniques.—It has long been recognized that morphology is an inadequate criterion on which to assess the genetic homogeneity of mammalian cell cultures. Failure to obtain growth of single cells and the necessity for a relatively large population of cells to initiate sustained growth raised the question of whether somatic cells might not be fundamentally different from the free-living microorganisms (11, 12). Until recently this requirement for multicellularity has prevented application of some of the techniques developed for the study of genetics in microorganisms. Sanford et al. (50) demonstrated that single isolated cells of strain L would proliferate in capillary tubes presumably as a result of restricted diffusion of medium around the cell. As a consequence, the first pure, or cloned, strain of mammalian

cells was isolated. The capillary tube method has been used successfully with other strains of cells (51, 52). Puck & Marcus (53) used a layer of x-irradiated HeLa cells ("feeder layer") which are unable to multiply but which exhibit sufficient metabolic activity to supply the factors required for the growth of single unirradiated cells. In subsequent experiments it was observed that by modifying the medium and techniques, a high proportion of single cells from several permanent strains (26, 35), as well as those from human biopsy specimens (54), will proliferate to yield clones when plated in Petri dishes in the absence of a feeder layer. This method permits studies with large numbers of colonies but does not exclude cross contamination among clones in a single dish. Various devices have been employed to insure the homogenicity of clones. Wildy & Stoker (55) used micromanipulative procedures described by Lwoff et al. (56), Hobbs et al. (57); and Pearson & Lagerberg (58) have employed a plasma coagulum, and Schenck & Mosko-

witz (59) isolated cells on small glass squares.

Growth of mammalian cells in suspension.—The fact that mammalian cells do not require a supporting framework of fibrin for optimal proliferation in vitro suggested to a number of investigators that cells could be propagated in suspension as are bacteria. Owens et al. (60) demonstrated that strain MB III of de Bruyn's mouse lymphosarcoma cells grew well when maintained in suspension by the "tumble tube" method. Primarily because of technical limitations, this method has not been widely used. Earle et al. (61) were successful in propagating strain L, of fixed tissue origin, in shake cultures and Graham & Siminovitch (62) used a "high speed" roller-tube apparatus for monkey kidney cells. Cherry & Hull (63) introduced the socalled "spinner culture" in which the suspension is stirred by means of a magnetic stirrer-bar suspended in the flask. Siminovitch et al. (64) have used the rotating culture method for many strains, including L. The initial studies done by Earle and his colleagues have been extended and additional strains of cells have been propagated in shake cultures (65, 66). Kuchler & Merchant (67) and Davis et al. (68) have also used the rotary shaker. The spinner culture has also been employed with several strains of cells (68, 69, 70). McLimans et al. (70) and Ziegler et al. (71) have propagated a number of strains successfully in fermentation apparatuses ranging from 5 to 20 L in volume. Graff & McCarty (72) have described a continuously regulated cytogenerator for the propagation of a variety of cells. The foregoing observations have opened the way for the propagation of mammalian cells on a scale necessary for many biochemical studies and indicate the feasibility of employing suspended cultures for the commercial production of viruses and perhaps even compounds of pharmaceutical interest. Certain special aspects of the nutrition of cells in suspension will be discussed in a subsequent section.

Miscellaneous.—The ever-increasing number of permanent strains of cells and clonal sublines has introduced new problems. Aside from the practical one of maintaining strains when not in use, difficulties may be experi-

enced as a result of microbial contamination and alterations in the physiological characteristics of stock strains. Successful preservation of cells at -70°C, has circumvented some of these. Scherer & Hoogasion (73) reported that strains L and HeLa could be stored at -70° C, in media containing glycerol along lines established with erythrocytes and tumor tissues. Stulberg et al. (74) and Baron & Rabson (75) have successfully stored additional strains of cells. Swim et al. (76) demonstrated that a variety of strains have a half life at -70°C. of from 1.5 to 3 years when stored under optimal conditions. It has been noted (76) that conditions for optimal survival vary among strains and also among variants and the parental stock from which they were isolated. Alterations in the properties of cells caused by freezing have not been observed but in view of the observations of Morgan et al. (77) with ascites tumor cells, it remains possible that as yet undetected changes may occur in tissue culture cells as a result of storage at -70°C. The literature on the short-term storage of cells at 5°C, and methods employed for the transportation of cells has been reviewed by Ross & Syverton (2).

Additional methods for cell culture have been developed to meet specialized requirements. These include cultures in sponge matrix (78), on glass wool (79), on collagen (80, 81), and on glass helices (82). Stable media prepared in dry form described by Swim & Parker (83) have many practical advantages including the standardization of solutions for long-term experiments. Specialized glassware (84) has contributed to the precision and convenience of research. Additional information on these items will be found in papers already cited in the text or in the review by Ross & Syverton (2).

CONTINUOUS PROPAGATION OF MAMMALIAN CELLS

Permanent strains.- The concept that mammalian cells can be maintained indefinitely in vitro is not new, as exemplified by the Carrel strain of chick embryo fibroblasts which was established in 1913 and propagated serially for 33 years (9). Paradoxically, it was also recognized very early in tissue culture studies that not all tissues contain cells with the capacity for rapid proliferation and that in most instances where growth occurs the cells eventually undergo nonspecific degeneration. As a consequence, much effort has been expended to establish conditions which will permit continuous proliferation of mammalian cells. Gey & Gey (23) have described methods for the long term cultivation of cells from both normal and neoplastic tissues and were successful in establishing a number of permanent lines (85, 86, 87). Strain HeLa (86, 88) isolated from a cervical carcinoma is a well-known example. Earle (89) obtained several lines of fibroblasts, including strain L, from adipose connective tissue of the mouse. Parker (90) has described other strains. In addition to the foregoing, many strains have been established in the past five years and some of these are indicated in Table I. Many strains of hemic origin have also been isolated; these include the 15 Detroit strains of epithelial-like cells of Berman & Stulberg from bone marrow of cancer-free patients (118), cells from bone marrow of cancer patients (118, 119), from

TABLE I

Some Permanent Strains of Cells Established from Normal and Neoplastic Tissues

Origin*	Designation†	Ref.	Origin*	Designation†	Ref.
Normal Human			Bovine emb. kidney	BEK-E	(107)
Amnion	FL-E	(91)	Bovine emb. lung	ND-E	(106)
Amnion	ND-M	(92)	Bovine emb. skin	BES-E	(107)
Amnion	A1 to A5-E	(93)	Monkey heart	SCH-E	(108)
Appendix	Chang E	(94)	Monkey kidney	MK1 to MK3-E	(99)
Conjunctiva	Chang E	(94)	Monkey kidney	ND-UC	(90)
Esophagus	ND-E	(95)	Monkey lung	MLu 1	(99)
Foreskin	FS4-F	(96)	Mouse liver	NCTC-721-E	(109)
Heart	ND-E	(97)	Ovine kidney	MDOK-E	(105)
Intestine	407-E	(98)	Rabbit kidney	RbK-E	(110)
Kidney	Chang E	(94)	Rabbit muscle	RM3-F	(27)
Liver	Chang E	(94)	Rabbit emb. kidney	ERK1-E	(99)
Liver	407-E; R-E	(98)	Rabbit emb. skin	RS1-F	(27)
Liver	ND-E	(95)	Rabbit testicle	RT6-F	(27)
Liver (emb.)	HLitE	(99)	Neoplastic Human		
Lung	To-E	(98)	Carcinoma-cervix	H.Ep.2-E	(111)
Nasal mucosa	DMB-E, DHov-E	(100)	Carcinoma larynx	H.Ep.2-E	(111)
Palate	ND-E	(95)	Carcinoma-lung	LAC-E	(75)
Skin	AU-E	(101)	Carcinoma-lung	MABEN-E	(112)
Skin	NCTC-1769-E	(102)	Carcinoma-lung	U.Va.130-E	(113
Skin (emb.)	MAFE-E	(98)	Carcinoma-lymph	O.Va.150-15	(110
Tonsil	T16-E	(103)	node	H.Ep.3-E	(111
Uterus	U12-F	(96)	Carcinoma-mouth	KB-E	(114
Animal			Reticuloendotheliosis		(115
Bovine kidney	RB1-E	(104)	Rhabdomyosarcoma		(111
Bovine kidney	MDBK-F	(105)			,
Bovine emb.	********	(.55)	Neoplastic Animal Carcinosarcoma	Walker 256	1116
diaphragm	ND-E	(106)		Sarcoma 180	(116
Bovine emb. kidney	ND-E	(106)	Sarcoma	Sarcoma 180	(117

* Emb. refers to embryonic tissue.

† ND—no designation assigned by investigators concerned; last letter refers to general morphologic characteristics where E=epithelial-like, F=fibroblastlike, M=mixed, and U=unclassified.

carcinomatous ascitic fluids (118), from lymphomatous pleural fluid (118), and from nonleukemic peripheral blood (120); the HERT 1 strain of Paul (121) from peripheral blood; strains J-96, J-111, and J-113 of Osgood & Brooke (122) from human leukemic blood; strains of McCulloch & Parker (123) from human leukemic leukocytes, from normal and leukemic bone marrow, and from the bone marrow of mice and rats; and lines from leukemic mice by Fischer (124) and Foley et al. (125). This increase in the number of permanent strains results partly from improved nutrition and methodology, but it also reflects the recent expansion of research on cell culture. Despite these advances, however, the methods currently in general use do not permit the continuous growth of cells from any tissue with a high degree of regularity.

Nomenclature of strains.—No basic pattern has been followed in naming strains of cells. Those indicated in Table I are designated with respect to the laboratory or tissue of origin, initials of investigators or tissue donor, or letters of the alphabet. These are occasionally followed by a strain number. The problem of a uniform system of nomenclature becomes more acute as additional strains and clonal sublines are added. Furthermore, it is becoming increasingly apparent that mammalian cells in vitro are subject to marked variations in properties just as are microorganisms. Recently, a committee which was assigned the task of establishing a cell nomenclature made the following recommendations (126): reference to a strain in a publication should include tissue and species of origin, whether normal or neoplastic, adult or embryonic, cell type, designation of strain and the clone number if applicable, and the reference to the original description. The designation should consist of not more than four letters indicating the laboratory of origin followed by numbers indicating the strain (XYZ 111, clone 29). Swim & Parker (127) have adopted a system of designating strains which takes cognizance of the fact that alterations in the composition of the medium or conditions of culture may result in the selection of a variant which differs significantly from the parental strain. Each time the cells are placed in a new environment the designation of the strain is changed accordingly to indicate that it may not retain the properties of the original line on continued cultivation.

Problems of infection in cell cultures .- The strict adherence to aseptic techniques and the use of antibiotics and cold storage methods are usually not sufficient in practice to exclude completely the loss of strains as a result of infection. Viruses (128 to 131) as well as amoebae (132) have been isolated from primary cultures of normal tissues obtained from apparently healthy animals under conditions in which the medium can be excluded as the source of the infecting agent. Gey (86, 133) has pointed out that the medium is a potential source of filterable agents and, on the basis of studies with the electron microscope, has suggested that cells in vitro may contain viruses or viruslike particles. This thesis is supported by the fact that in certain instances when cultures are purposely infected, both the cells and virus may coexist for long periods (6). Recently, filterable forms of bacteria, the socalled pleuropneumonialike organisms (134), have been isolated from a number of strains of cells (135 to 138). On the basis of available data, it is not possible to assess the frequency of these infections or the effectiveness of antibiotic therapy in eradicating the organisms. Ascertaining the extent to which inapparent infections of mammalian cells with intracellular agents may alter their physiological characteristics promises to be an exciting area of future research in cell culture.

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CELL CULTURE NUTRITION

Introduction.—Nutrition continues to be the most important limiting factor in the expansion of cell culture applications. Media prepared from

plasma, serum, and tissue extracts are satisfactory for the long-term cultivation of many cells, but their complexity and variability make it difficult to identify individual growth factors. For a comprehensive treatment of the earlier literature the reader is referred to reviews by Waymouth (139) and

by Morgan (140, 141).

Chemically defined media.—Because of the difficulties involved in identifying the active components of serum and tissue extracts, many studies have been directed toward devising a chemically defined solution by the stepwise addition of potential nutrients to a defined basal medium. The object of these experiments has been to develop media which are adequate for the continuous propagation of cells and then by a process of elimination to determine the essential constituents. Representative examples of these are as follows: solutions 199, M150 of Morgan et al. (142, 143), 703 and 858 of Healy et al. (144, 145), V-614 of Fischer et al. (146), WII of White (147), CW 47/2 of Waymouth (148) and MS of Scherer (149). These so-called "synthetic" solutions are maintenance media and do not permit the sustained growth of cells. The interval of survival is a function of both the medium and the strain of cells. Defined solutions, however, provide excellent media for the continuous growth of a variety of cells when fortified with serum or serum and embryo extract. Maintenance media have also made a major contribution to virology by virtue of the fact that they preserve the capacity of cells to support viral multiplication. The advantages of protein-free media for virus studies are discussed by Ross & Syverton (2). Evans et al. (150, 151) and McQuilkin et al. (152) developed a defined medium designated as NCTC 109, which has permitted the serial propagation of strain L-2071 for several years. Strain 2071 (151, 152) was obtained by "adapting" L-929 to medium 109. The 2071 line of L cells appears to be a nutritional variant of L-929. As might be anticipated, the inoculum size is a critical factor in preparing cultures of 2071 (153). Growth of additional strains in 109 has not been reported. Despite the fact that 109 does not fulfill the requirements of a practical medium, it has served to demonstrate clearly that protein is not an absolute requirement for all mammalian cells.

Amino acid requirements.—Recent investigations on the specific nutritional requirements of cells in vitro have been conducted along lines introduced by Fischer (154) who employed a defined medium supplemented with dialyzed plasma as a means of identifying certain growth factors. The success of recent studies has been largely dependent on the introduction of quantitative methods, on improvements in defined media, and on the availability of strains which proliferate in defined media fortified with dialyzed serum. It has thus been possible to define the requirements of a number of strains, exclusive of the factors supplied by the dialyzed serum, by a process of elimination and the application of quantitative techniques.

The amino acids required by several permanent strains and by primary cultures of monkey and chick embryo cells are indicated in Table II. The amino acid requirements of man and chick, as established by feeding experi-

TABLE II

Amino Acid Requirements of Various Cells in vitro Compared with the Requirements of the Intact Chick and of Man

L-Amino acid	Strain HeLa* (42) 155	Strain U12 ^a (44)	Strain L* 155 156	Walker 256* 157	Strain RM3* (43)	Monkey kidney ^b 158	Monkey testicle ^b 159	Chick heart ^e 160	Man 161	Chick 162
Asparagine	_d	_	_	+	_	-	_	-	_	_
Arginine	+	+	+	+	+	+	+	+	-	+
Cyst(e)ine	+	+	+	+	+		+		-	-
Glutamine	+	+	+	+	+	+	+	_	-	-
Glycine	-	-	-	_	-	+	+	-	_	+
Histidine	+	+	+	+	+	+	+	+	-	+
Isoleucine	+	+	+	+	+	+	+	-	+	+
Leucine	+	+	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+	+
Methionine	+	+	+	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+	+	+
Serine	-	_	_	_	+	-	-	-	_	-
Threonine	+	+	+	+	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	+	_	_
Valine	+	+	+	+	+	+	+	+	+	+

^a Media used to determine these requirements contained various proportions of dialyzed serum.

b Requirements determined in first passage in vitro.

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^e Primary cultures of chick embryo heart fibroblasts. Requirements determined by the depletion technique (163).

d+ indicates essential: - indicates nonessential.

ments, are included for purposes of comparison. The reliability of the methods is exemplified by the fact that strain L requires the same amino acids in a defined medium (164, 165) as those found essential in the presence of serum protein (156). Eagle (166) found that dipeptides containing essential amino acids substitute quantitatively for the single compounds. None of D-enantiomorphs of the essential amino acids permit growth (42, 43, 44, 156). The various cell species possess only a limited degree of individuality with respect to the concentration of individual amino acids which permit optimal growth.

In addition to the amino acids required for nitrogen balance in man (161), primary monkey cells as well as established strains of mammalian origin require arginine, cystine, glutamine, histidine, and tyrosine. This raises the question whether or not some of these amino acids are essential for a variety of cells in vivo and are synthesized in some organ and supplied to the rest of the body. Alternatively, cells in vitro may be capable of synthesizing certain amino acids from precursors not supplied in minimal media but which may be available in vivo. Cystine might be an example since Eagle (158) has stated that reduced inorganic sulfur compounds will replace cystine. Morgan & Morton (167) have found that the cyst(e)ine requirement of chick embryo cells for survival in a defined medium is highly specific. Morgan & Morton

(160, 168) reported that glutamine is not required for the survival of depleted (163) chick embryo cells and that glutamine accumulates in the medium. This raises the question whether or not the nutrients required for limited periods of survival may differ from those which are needed for growth, since glutamine has long been considered essential for the prolifera-

tion of chick embryo cells (146, 154, 169).

The mammalian cells examined to date are remarkably similar in that all require the same thirteen amino acids, but it should be noted that several strains have additional requirements (Table II). Glutamic acid replaces glutamine quantitatively in the case of monkey kidney (158) and testicular (159) cells, whereas it promotes the growth of HeLa (170) only at high concentrations, and strains L (170), RM3 (43), and U12 (44) fail to respond to this amino acid. An explanation for the dual requirement of the Walker 256 for amide-nitrogen is not apparent. Similarly, the role of glycine in the nutrition of monkey kidney needs clarification since it is synthesized by these cells (158). Investigations on the metabolic interrelationships between amino acids are required to resolve these apparent discrepancies. It should be noted that the technique of deleting single amino acids has certain limitations because of the complex interrelationships between these compounds as exemplified by studies with bacteria (171) and Tetrahymena (172). Haff & Swim (43) and McCoy et al. (157) have observed that several nonessential amino acids stimulate the growth of cells in minimal media. Evans et al. (173) have observed that the growth rate of L-2071 is reduced significantly when accessory amino acids are omitted from medium 109. Recent advances in amino acid nutrition should be regarded as preliminary steps in defining the precise needs of mammalian cells for optimal proliferation in vitro. It is emphasized that other strains may require amino acids not indicated in Table II. The extent to which differences in amino acid requirements will be useful for purposes of differentiating strains is merely a topic for speculation at the present time.

Vitamins.—The vitamins required by several strains for growth in media containing dialyzed serum have been determined. The studies done by Eagle (174, 175) with HeLa and by Swim & Parker (176) with U12 indicate that choline, folic acid, inositol, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin are essential. HeLa and U12 resemble numerous other strains of human cells in their requirement for inositol (175, 177, 178). Haff & Swim (179) found that strain RM3 does not require inositol and choline under comparable conditions. Inositol does not appear to be required by L (175) but the other vitamins indicated for U12 and HeLa are essential (174). These observations do not indicate that inositol is required specifically by human cells since it is essential for mouse Sarcoma 180 (175). Strains U12 (176) and RM3 (179) resemble certain microorganisms (180) in that pyridoxal is replaced by nonessential amino acids. The data are consistent with the interpretation that amino acids reduce the requirement for pyridoxal to a minimal level since other components of the medium, notably dialyzed

serum, may contribute traces of this compound. Various derivatives of the essential vitamins permit the growth of strains U12 (176) and L (181). Differences have been noted between U12 and HeLa in their response to certain cofactors (176) and strains U12, HeLa, L, and RM3 are dissimilar with regard to the concentration of various vitamins which are required for maximal proliferation. It is not possible to assess the significance of quantitative differences among strains because of uncertainties introduced by the use of different methods and reagents. It should be noted that the foregoing data indicate only minimal requirements since additional vitamins may be contributed by the dialyzed serum (137). There are numerous additional reports (139) on the effects of vitamins on the growth of cells *in vitro*. However, the media used generally contained sufficient quantities of undefined constituents to render unequivocal demonstration of specific requirements impossible.

Inorganic ions and bicarbonate.—Media are invariably prepared in a socalled balanced saline solution. The most widely used formulae (23, 89, 182, 183) are based on the assumption that salt solutions should approximate the inorganic composition of body fluids. Eagle (184) has shown that strains L and HeLa require various concentrations of Na+, K+, Mg++, Ca++, Cl', and PO4" for growth in a minimal medium. Shooter & Gey (185) demonstrated that Ca⁺⁺, Mg⁺⁺, and K⁺ are essential by treating serum with ion exchange resins. Owens et al. (186) have described the effects of Ca++ and Mg++ on the growth and morphology of strain MBIII. Trace elements are almost certainly necessary for cells in vitro, but there is little definitive information on this topic (139, 141). Harris (187) found that bicarbonate was essential for the outgrowth of cells from explanted chick embryo tissue. Swim & Parker (188) have shown that carbon dioxide is essential for six strains of fibroblasts of diverse origin, and Geyer & Chang (189) demonstrated that HeLa requires bicarbonate. The results of these recent studies are not in accord with the conclusion of Harris (187) that the principal function of bicarbonate is in the maintenance of the appropriate intracellular pH. Many mammalian cells resemble microorganisms in that sufficient CO2 is produced metabolically to satisfy their nutritional needs as indicated by the fact that growth in closed flasks is unaffected by the omission of bicarbonate (188, 189). The data of Swim & Parker (188) are consistent with the view that a closed system is required for the practical use of fixed buffers (33, 190) as a substitute for the conventional carbon dioxide-bicarbonate system.

Miscellaneous.—D-Fructose, D-mannose and maltose have been found to substitute for D-glucose in the outgrowth of cells from explants of chick embryo tissue (191, 192, 193). Conflicting results were obtained with D-galactose. Chang & Geyer (194) and Reuchert & Mueller (195) reported that either fructose, maltose, mannose, or galactose will support the growth of HeLa. D-Ribose has generally been considered to be inactive (191 to 194). Eagle et al. (196) have recently examined nine strains of cells with regard to their ability to proliferate on various carbohydrates. A number of compounds

were active and the concentration of each which permitted growth varied over a wide range. Ribose was effective for some of the strains tested but only in a very narrow range of concentrations. The growth response to galactose was variable. Pyruvate exerts a sparing reaction on glucose and certain other sugars (194, 196). It is difficult to assess the significance of the fact that a variety of di- and polysaccharides support the growth of cells (192, 194, 195, 196) since these are readily hydrolyzed by enzymes in serum (192, 195). Chang (197) has recently isolated variants of strain HeLa which are characterized by their ability to grow on ribose or xylose, sugars which are inactive for the parental strain under the conditions employed. He has suggested that the variants utilize these compounds in lieu of glucose. Swim (198) has isolated variants of U12 which proliferate in media containing sugars which are inactive for the parental strain. These are all characterized by their ability to proliferate in unusually low concentrations of glucose. It is clear from these findings that the degree of purity of the so-called Reagent Grade sugars should be considered in interpreting data relevant to the nutritional requirements of cells.

Requirements for purines and pyrimidines or their derivatives have not been demonstrated. Various members of this group, however, have been added to maintenance media with beneficial effects (141). Similarly, various

lipides have been used (139, 141).

The growths of cells in suspension presents a number of practical problems. Earle et al. (61) introduced the use of methylcellulose to increase the viscosity of the medium as a means of maintaining cells in suspension with a minimum of agitation. Essential amino acids (199) and glucose (200) may be growth-limiting by virtue of the fact that they are utilized rapidly by cultures of high population density. It is not surprising, therefore, that media designed for stationary cultures are not satisfactory for the sustained growth of cells in suspension when the simple expedient of periodic addition of fresh medium is employed (201). Frequent replacement of the medium is practical, although undesirable, with the usual stationary cultures, but imposes severe limitations on large-scale suspended cultures. McLimans et al. (69) have attempted to circumvent this difficulty by adjusting the concentration of essential amino acids and vitamins in the medium on the basis of cell population. Earle et al. (65) increased the glucose concentration in media used for shake cultures. Thomas et al. (202) found that the interval between fluid replacements with strain L, in a conventional medium, could be prolonged by the periodic addition of arginine. Cultures are frequently gassed with CO₂-air mixtures (61, 65, 66, 70). The results of a number of studies (62, 64, 70, 71) indicate that this procedure may not be necessary in all instances if the pH is adequately controlled by other means. In this connection, the studies of Cooper et al. (203) indicate that reduced oxygen tension is beneficial for the proliferation of monkey kidney cells in submerged cultures.

The fact that single mammalian cells, just as certain bacteria, fail to proliferate under conditions which permit the rapid growth of a relatively large population is well documented (50 to 53). The cloning techniques of Puck and his colleagues (26, 35) have made it possible to examine the nutritional requirements of single cells. Sato et al. (204) found that the addition of cholesterol to a medium prepared with well dialyzed serum increased the cloning efficiency of HeLa. Neuman & McCoy (205) achieved similar results with the Walker 256 by fortifying the medium with pyruvate or other α-ketoacids. Neuman & Tytell (206) found that a number of purines stimulate the growth of Walker 256 cells in isolated colonies. These are additional examples of the individuality of the Walker 256 in regard to its nutritional requirements. Recently, Lockart & Eagle (207) reported that accessory amino acids, notably serine, increase the cloning efficiency of several strains of cells. These latter findings are in agreement with earlier observations (43, 157, 173) which demonstrated the beneficial effect of accessory amino acids on the growth of cells in minimal media.

Bacteriological media have always been of interest as a potential source of nutrients for mammalian cells, and a large section of the early literature on tissue culture is devoted to this topic (14, 139). Attention has recently been redirected to bacteriological media in search of economical substitutes for complex amino acid-vitamin mixtures. Some examples of those which have been used recently are as follows: lactalbumin hydrolysate (208), peptone (209, 210), tryptose phosphate broth (211), and skim milk (212).

A review of some of the factors involved in the suitability of various sera for cell culture can be found in recent papers of Cailleau et al. (213, 214).

Undefined components.—Recent speculation whether dialyzed serum promotes growth by virtue of simple compounds adsorbed to the protein (155), or whether a specific protein is the active principle (215), has not been resolved. There is no general agreement on the activity of Cohn fractions from serum (155, 216). Fisher et al. (217) isolated an α -globulin-containing fraction from serum which promotes the attachment of HeLa cells to glass. The activity of this material is enhanced by the addition of albumin. Neither fraction singly nor in combination promotes growth. Lieberman & Ove (218, 219) have isolated and purified a similar attachment factor which appears to be a glycoprotein. The purified protein fraction fortified with peptone and albumin permits only limited growth. These same authors (220) have observed recently that catalase enhances the survival of several strains in a defined medium as determined by plating efficiency. An explanation for this effect is not apparent since the same strains contain high levels of catalase (221).

Little attention has been directed recently to the nature of the growthpromoting factors in embryo extract because of the general availability of cells which do not require it. Nevertheless, many strains require embryo extract even in complex defined media fortified with serum (54, 96, 110).

Kutsky & Harris (222) and Katsuta et al. (223) have isolated a nucleoprotein fraction which is active for primary cultures of chick embryo fibroblasts. Harris & Kutsky (224) found subsequently that most of the activity of the nucleoprotein fraction resides in the protein moiety.

CELL CULTURE METABOLISM

Introduction.—Only in recent years has it been possible to propagate established strains of cells in semidefined media on a scale which is practical for most biochemical studies. Systematic investigations of the metabolic characteristics of cell lines are thus in their infancy, and most of the literature on tissue culture biochemistry is concerned with explanted tissues. Limitations imposed by space, however, make it necessary to restrict the discussion to studies with established cell strains.

Comparative biochemistry of cells .- The availability of established strains of diverse origin provides the opportunity for investigation of such fundamental problems as metabolic relationships between dedifferentiated cells in vitro and the constituent cells of the original tissue, comparative biochemistry of cell lines with respect to their source and whether derived from normal or neoplastic tissues, and biochemical aspects of the genetics of somatic cells. Perske et al. (225) tested a strain of human liver cells for four enzymes which are reputed to be distinctive of hepatic parenchyma. Only glucose-6phosphatase was detected. The results obtained by Westfall et al. (226, 227) were likewise not consistently indicative of any general metabolic relationship between a variety of strains and the corresponding tissues of origin. Additional strains have been compared with respect to a variety of enzymes. Lieberman & Ove (221) did not find significant differences in the specific activities of thirteen enzymes between strains of appendix, liver, lung, and HeLa. Eagle et al. (196) found that the level of activity and specificity of hexokinase was very similar in a number of strains. Moore & Wroblewski (228) observed that a variety of lines from both normal and neoplastic tissue differed markedly in their lactic dehydrogenase activity but were similar with respect to transaminases. It is not possible to evaluate the significance of the foregoing data. Cells which characterize a tissue biochemically in situ may not proliferate in vitro, and thus many established lines may represent the progeny of other species of cells present as a minority in the tissue of origin. Any consideration of the relationships between cell lines or various strains and the tissue of origin must take into account the variations which may occur in vitro. The observations of Westfall et al. (227) provide a striking illustration of such changes. Strains 1745 and 2050 of mouse cells were obtained from the cloned 1742 lines. Strain 1745 contained twenty times and strain 2050 had four hundred times the arginase activity of the parental 1742. Studies of the comparative biochemistry of cells in vitro are further complicated by the paucity of definitive information on metabolic changes which occur when cells are deprived of the regulating factors available to them in vivo. Recently, Lieberman & Ove (221) found that the specific activity of several enzymes in dispersed monkey kidney cells change markedly during the first week or so in vitro. In experiments of this type it is difficult to exclude the possibility that changes in the population may result from the growth of a particular type of cell with the concomitant degeneration of others. It should be noted also that cells may maintain certain biochemical traits for long periods in vitro which are considered distinctive of the tissue in situ. Jones et al. (229) and Waltz et al. (230) observed the production of gonadotrophin by cultures of hydatid mole for periods of one to fifteen months. Similarly, Grossfeld (231) and Castor (232) have found that several explanted tissues produce hyaluronic acid for extended periods. With the availability of established lines of cells it has been possible to initiate studies of factors affecting their metabolic activity. De Mars (233) found that the specific activity of glutamyl transferase in HeLa increases when glutamic acid was substituted for glutamine in the growth medium. This increase in transferase was almost completely suppressed in a medium containing both glutamic acid and glutamine. When cells grown in glutamic acid were returned to a medium containing glutamine or glutamic and glutamine the specific activity of the transferase decreased. Eagle et al. (234) found that a variant strain of HeLa converts phenylalanine to tyrosine. The HeLa hydroxylase, unlike that in liver (235), was completely inhibited by the presence of tyrosine in the growth medium. Lieberman (236) observed that exposure of intact cells or extracts of strain L to DPN for 45 min. reduces their DPNase activity to undetectable levels. When cells previously exposed to DPN were placed in a DPN-free medium, the DPNase activity gradually returned to the initial level over the first three to four cell generations. Nicotinamide is more effective than DPN in suppressing enzyme activity and the author suggests that DPNase is inactivated by the formation of an enzyme nicotinamide complex. From studies with bacteria, the concept has gradually emerged that an exogenous supply of metabolite which can enter the cell will generally interfere with the intracellular synthesis of that compound (237, 238). In addition to a simple mass law effect, it has been demonstrated that the presence of a metabolite may either block the synthesis of an enzyme in the biosynthetic pathway (239) or inhibit the activity of at least one such enzyme (240). The recent studies of the effects of glutamine (233), tyrosine (234), and DPN (235) on enzyme activities in established strains of mammalian cells indicate that a similar system of metabolic controls may be operative although additional experiments are required to establish the mechanisms involved. Similarly, it does not seem unreasonable to expect in future that the results obtained with permanent cell lines and even bacteria may provide clues both to the nature of some of the biochemical changes involved in cells during the transition from in vivo to in vitro conditions, and to some of the alterations in enzyme levels which have been observed in tissues and organs in situ (241).

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Carbohydrate metabolism.—Established cell lines from normal tissues have many features in common with those derived from neoplastic sources, and with malignant cells per se. Many criteria have been used in attempts to evaluate the status of various strains of cells with respect to malignancy. Most metabolic experiments have been influenced by the Warburg (242) theory of cancer which states that the fundamental distinguishing biochemical characteristics of malignant cells are a relatively high glycolytic activity and impaired respiration. Numerous studies (85, 243 to 249) have been conducted on the respiration and glycolysis of established strains from both normal and neoplastic sources. No general metabolic features have been noted which are capable of distinguishing cells with respect to their origin. Most of the cell lines appear to fall into the category classified as malignant (242, 250). In this connection, it is of interest that the glycolytic activity of the high-malignancy line of strain L was found to be somewhat greater than that of the low-malignancy strain (242). Similar results were obtained when a tumor-producing line of rat fibroblasts was compared with the nonmalignant parental strain (85). Several possible explanations may be considered in accounting for the similarity in the range of metabolic data for cells in vitro irrespective of their source: (a) all cells capable of continuous growth in vitro are of the malignant type; (b) cells in vitro adopt a common type of glucose metabolism which would tend to obscure metabolic differences existing in vivo; and (c) Warburg's criteria of malignancy do not apply to cell culture systems. Although these criteria were described three decades ago, the conflicting views on the basic metabolic differences between normal and malignant cells have not been resolved (250). Similarly, any attempt to define cells derived from normal tissues as either normal or malignant invites controversy on one or more of the currently available criteria.

The availability of permanent strains makes it possible to examine the factors which affect the carbohydrate metabolism of mammalian cells in a more definitive manner than is possible with tissue slices and similar preparations. The lack of correlation between carbohydrate utilization and growth has been observed by a number of investigators (195, 196, 200, 226, 245, 247). The results of several studies (200, 245, 251) indicate an inverse relationship between population density and glucose utilization. It has also been observed that the rate at which a given sugar is metabolized is a function of its chemical structure (195, 196) and varies directly with its concentration (196). Leslie et al. (248) found a direct relationship between glucose "oxidized" and growth. The glucose oxidized was calculated by subtracting the lactate, in glucose equivalents, from the hexose utilized. Under conditions of relatively constant glucose utilization, the rate of lactate production was rapid initially and subsequently declined. These investigators suggest that in fresh medium lactic acid leaks from the cells, and that it is not utilized to an appreciable extent until a sufficient concentration is attained in the medium to maintain appropriate intracellular levels. This "leakage" phenomenon could account for the decreased respiration observed after replacement of the medium (252), and for the changes in the ratio of lactate to CO₂ with time observed by Suchny et al. (251). On the other hand, the leakage phenomenon is not in accordance with the finding that optimal growth of several strains is obtained with minimal utilization of D-galactose under conditions in which very little lactate accumulates (195, 196). It would appear from the data on growing cells that the amount of carbohydrate metabolized, the efficiency of its utilization, and the extent of aerobic glycolysis are all interrelated. Eagle et al. (196) have recently proposed that the most important single determinant factor is the rate at which the hexose becomes available to the cell as hexose-PO₄.

Few experiments have been conducted on the intermediary metabolism of carbohydrates by established lines. Barban & Schulze (253) found all of the enzymes of the tricarboxylic acid cycle in extracts of HeLa. The presence of the required enzymes does not in itself indicate that the cycle functions as an organized sequence of reactions in intact HeLa cells. The quantitative significance of the tricarboxylic acid cycle in carbon transformations in terminal respiration of established cell lines remains to be determined. Hiatt (254) found that the isotope distribution in D-ribose isolated from the RNA of HeLa and of a strain of liver cells grown on glucose labeled in various positions, was consistent with the functioning of the hexose monophosphate pathway in these cells. Graff & McCarthy (255) found in tracer experiments that glucose carbon contributes to RNA, DNA, and protein during the growth of strain L.

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Amino acid metabolism.—As in most areas of cell culture metabolism at the present time, the data concerning amino acid metabolism are fragmentary. The patterns of amino acid changes in the culture medium have been studied with established lines (199, 256, 257) and with primary cultures (168, 258). Piez & Eagle (259) have found that several strains do not differ significantly with respect to the amino acid composition of total cell protein and the free amino acids of the intracellular pool. Omission of essential amino acids from the medium did not alter the pool of other amino acids. The intracellular concentration of most amino acids was significantly greater than that of the corresponding compound in the medium. The studies of Levintow et al. (260) with growing cultures indicate that glutamic acid and glutamine are incorporated into protein independently, and that the carbon chain of glutamine can serve as a precursor of aspartic acid and proline. Salzman et al. (261) found that glutamine-C14 and glutamic acid-C14 are incorporated into pyrimidines to a much greater extent than into purines during the growth of HeLa. The addition of nonessential amino acids, including aspartic, suppressed the incorporation of glutamine carbon into purines. The results indicate that HeLa differs from other systems (262) in that the amide nitrogen of glutamine was the source of one nitrogen atom of the pyrimidine ring and of the cytosine amino group. In connection with the latter it should be noted that although HeLa is permeable to ammonia, it is not a precursor of any of the nitrogen atoms of the purines or pyrimidines.

Metabolic activity of cellular constituents.—Some years ago Schoenheimer (263) proposed that all constituents of the animal body, whether structural or functional, were undergoing continuous turnover in which the synthesis of tissue components was exactly balanced by their degradation. The significance of turnover rates as determined in animals or tissues containing a variety of cell types is open to debate. The results of isotope experiments could be explained by cell turnover and by secretion and replacement; this would not be turnover in the sense of synthesis and breakdown as an exclusively intracellular process. Cell culture and other homogenous populations of freeliving cells provide systems which permit a more critical examination of the stability of cellular constituents. The results obtained by Eagle et al. (234) on the incorporation of phenylalanine-C14 into both nonproliferating and growing cells of mammalian origin were indicative of protein turnover. In a subsequent study (264), it was observed that when prelabeled cells were planted in an isotope-free medium, protein C14-amino acid residues were replaced by the corresponding unlabeled amino acid from the medium at the rate of about one per cent per hour. These data were consistent with the results of studies on amino acid incorporation. Data obtained by coupling labeled and unlabeled cultures are consistent with the interpretation that protein renewal was largely an intracellular process and not the result of cell turnover or protein secretion followed by degradation and resynthesis. These experiments provide no information as to the mechanism of amino acid replacement in protein. Significant differences were not observed between primary cultures and strains which had been propagated for years. Protein renewal occurred in both growing and "resting" cells at the same rate. In this respect mammalian cells differ from bacteria in which similar turnover has been observed in nonproliferating cells (265, 266), but not under conditions of growth (266). It should be noted that this difference may be relative rather than absolute because of the problems in detecting turnover in bacteria (266). The rate of protein renewal observed in established cell lines is of the same order of magnitude as has been reported for Erlich ascites cells (267, 268, 269).

Studies analogous to the foregoing have been conducted to ascertain the metabolic stability of DNA and RNA. Healy et al. (270) and Graham & Siminovitch (271, 272) observed that the P³² of both DNA and RNA was retained completely by pre-labeled cells during exponential growth in a P³²-free medium. Cheng & Mueller (273) observed that HeLa cells labeled with thymidine-C¹⁴ retained the isotope in DNA during logarithmic growth in a medium containing thymidine, but it was lost slowly under conditions of minimal growth. Thompson et al. (274) found that when L cells, pre-labeled with formate-C¹⁴, were permitted to grow slowly in a C¹⁴-free medium, the isotope was progressively lost from the RNA but was retained by the DNA. In the presence of exogenous thymidine there was a minimal loss of C¹⁴ from DNA. Thompson et al. (274) reconcile the differences between their results and those of the Toronto group by suggesting that isotope is

retained in the RNA of rapidly growing cells to a greater extent than in cells proliferating more slowly. The results of recent studies with bacteria summarized by Barner & Cohen (275) tend to support this view. The data of Thompson are in agreement with current concepts inasmuch as they indicate that DNA is metabolically much more stable than RNA. This correspondence with other systems, however, in no way alleviates the need for more experiments to clarify the discrepancies in the data obtained with strain L.

Metabolism of steroid hormones .- It has been known for some time that liver inactivates a variety of steroid hormones, but only recently has evidence been presented for the extrahepatic metabolism of these compounds (276, 277). Recent studies with established cell lines indicate that cells in vitro also have the capacity to metabolize steroids. Sweat et al. (278) and Grosser. Swim & Sweat (279) have found that strain U12 of human uterine fibroblasts converts both progesterone and cortisol to a variety of steroid products. Progesterone yielded 4-pregnene-20β-ol-3-one, 4-pregnene-20α-ol-3-one, allopregnane-3,20-dione and several unidentified steroids. Six products of cortisol metabolism have been identified. Berliner et al. (280) observed that strain U12 converts corticosterone to 4-pregnene-11\beta-20\beta-21-triol-3-one. Johnson & Reneal (281) reported that a number of cell strains of epithelial-like morphology converted cortisol to several unidentified products. It has also been found that a variant strain of U12, which is resistant to the growthinhibiting action of cortisol (282), metabolized this compound at a more rapid rate than the parental strain (279). Berliner et al. (283) demonstrated that U12 synthesized cholesterol from acetate. In this connection, it is of interest that nonsteroid products or progesterone-C14 and cortisol-C14 metabolism by U12 have not been detected.

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EVOLUTION OF CELLS IN VITRO

Even with improved techniques and media, only a small proportion of primary cultures yield cells which are capable of sustained growth in vitro. The common experience of many investigators indicates that the early cultivation of cells usually follows a characteristic course which can be conveniently divided into three phases. In phase I the cells proliferate rapidly after an initial lag and usually can be transferred serially without difficulty. Phase II is characterized by a decrease in multiplication to a point where it usually ceases entirely and the cells are eventually lost as a result of nonspecific degeneration. Infrequently, a third stage is recognized by the appearance of actively proliferating cells in phase II cultures. The transition from phase II to phase III may follow one of several patterns. (a) The degenerating cells are gradually replaced by actively growing ones which resemble the original cells in morphology and growth pattern (27, 102, 109). (b) An occasional colony develops which contains cells indistinguishable from those of Phase I on the basis of gross morphology and growth pattern (27, 99). (c) Cells of the same basic type with modified morphology and pattern of growth appear

in the culture (85, 89, 90, 96, 98, 123, 284, 285). (d) Islands of epithelial cells appear in phase II cultures which formerly appeared to be composed exclusively of fibroblasts (93, 100, 110, 119, 120). The duration of each of the three phases is extremely variable and does not appear to be related to the tissue of origin. The transition from phase I through phase III may be completed in less than a month (98, 99, 120) or may require almost a year (102). Phase I may terminate in the first or second passage (96) or be prolonged for more than six months (96, 102). Cultures may persist in phase II for many months before the appearance of phase III cells. Of particular significance is the fact that the phase III cells can be propagated indefinitely in a variety of media regardless of the evolutionary pattern followed in their origin from phase II. It should be emphasized that phases I and II represent the usual pattern, while phase III is a relatively rare event. The fact that strains usually do not proceed beyond phase II does not appear to be related to the source of tissue, since it has been observed with cells from (human) adult and embryonic tissues of animal origin (27, 97, 286) and from neoplastic tissues (88, 246). Morphologic alteration or "transformation" of cells in vitro has been observed and studied for many years by Parker (90, 287), Gey (85), Earle (89, 288) and their colleagues and has been noted recently by many investigators. It should be noted that alterations in individual strains range from those exhibiting essentially the whole spectrum of changes to those in which modifications have been minimal.

In addition to altered morphology, cells of phase III may differ from those of phases I and II in their virus spectrum (96, 287, 289). Several strains of "transformed" cells have been shown to be malignant by animal inoculation (85, 288, 290). On the other hand, wide variations in tumor-producing activity exist among strains (288, 291), and some permanent lines of murine cells do not appear to possess this capacity (85). The fact that many established strains are remarkably similar in many respects, whether derived from normal or neoplastic tissues, is indicative of the malignant potentialities of strains from normal human sources. Immunological incompatability between species has limited direct experimentation (292, 293). To circumvent this problem, cortisone-treated and x-irradiated animals (246, 292, 294) and normal hamsters (296) have been used in attempts to obtain information on the malignant characteristics of various strains of human cells. The results are by no means unequivocal because of the uncertainties involved in experiments with heterologous hosts (246, 297). Of the many problems associated with the serial propagation of cells from normal tissues, none is more challenging or is receiving more attention than that of in vitro "transformation" and its relationship to malignancy.

In addition to changes in morphology and growth pattern, karyotypic alterations have been observed. Recently, the chromosomal constitution of a variety of established lines has been studied in some detail as a means of comparing them and of obtaining information on the cytogenetics of somatic

cells, particularly as it relates to their "adaptation" to in vitro conditions. Hsu & Moorhead (298) found several strains of neoplastic and of normal origin to be heteroploid with very few or no diploids and tetraploids. The modal chromosome numbers varied from 60 to 80. Levan (299) found strains of both fibroblastic and epithelial-like morphology from both normal and neoplastic human tissue to be uniformly heteroploid. Individual strains varied from hypodiploid to near tetraploid, with the majority of strains exhibiting stemline numbers in the 50 to 80s range. Additional studies by Ruddle et al. (300), by Westwood & Titmuss (301), and by others (302 to 305) indicate that established strains are usually heteroploid and exhibit wide variations in their stemline modes. Changes in the chromosome constitution of established lines are not confined to mere shifts in ploidy. Studies of chromosome morphology (295, 299, 303, 305, 306) have revealed drastic alterations in chromosomal alignment, indicating that considerable genetic rearrangement has occurred in cells during the transition from in vivo conditions to sustained growth in vitro. As with so many other characteristics already noted, established strains resemble malignant cells cytologically (305, 307) and exhibit the same karyotypic variations regardless of whether they are of neoplastic or of normal origin. Somatic aneuploidy is not restricted to established strains and has been observed in primary cultures (308) and also in vivo (305). Hsu & Moorhead (305) have pointed out that insofar as chromosomal abnormalities are concerned, the differences between normal and malignant cells appears to be quantitative rather than qualitative. In order to understand the significance of the chromosomal patterns of established lines in relation to their capacity for sustained growth, it is of importance to obtain information regarding alterations in karyotype as a function of time and conditions of culture. Hsu et al. (309) demonstrated, by following a primary culture cytologically through many subcultures, that the ploidy of cells from normal human tissue changed during serial cultivation. Ford & Yerganian (306) and Tjio & Puck (310) observed changes in chromosome number and morphology of several strains from the Chinese hamster on serial cultivation. Tjio & Puck (310) and Puck et al. (311) on the other hand, have reported that a wariety of human cells can be cultivated serially for at least 5 to 10 months in the euploid condition in a medium containing fetal calf serum instead of embryo extract. It should be noted that the growth rate of the euploid cells after many months in vitro was about one-tenth that of the initial cultures. It remains to be determined whether these cultures will yield euploid cells capable of sustained and rapid growth comparable to that of aneuploid strains from the same source. It is of interest in this connection that an euploidy was first noted in the hamster cells within a month in vitro, and that after 10 months these cells continued to proliferate at the original rate (311). Puck et al. (310, 311) have stressed the importance of the medium in propagating euploid cells serially, and in one instance it was observed that human cells in embryo-extract medium were highly aneuploid

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after two months in vitro. Puck (312) reported that x-irradiation of euploid human cells produces chromosomal anomalies whose nature and frequency are a function of the dose. These data were considered to indicate that the primary process resulting in loss of reproductive capacity of cells by x-irradiation is damage to the chromosomes.

Karyotype analysis of cell lines has focused attention on the important problem of cross contamination of strains within a given laboratory. Ford & Yerganian (306) reported that a strain of hamster cells had been contaminated with a human cell carried in the same laboratory. The appearance of the contaminant would have been indistinguishable from some of the "transformations" already described except on the basis of immunological and cytological criteria. Hsu & Klatt (303) interpreted the results of cytological studies to indicate that the FF strain of human cells was in reality a subline of the L-929 mouse fibroblasts. On the basis of chromosome and transplantation studies, Rothfels et al. (295) concluded that many of their altered cell lines had arisen by contamination with strain L. These investigators also reported that the ERK1 strain of rabbit kidney was cytologically indistinguishable from HeLa. It should be noted that ERK1 supports the multiplication of polio virus, whereas primary cultures from the same source do not (313). The divergent results of several investigations on the immunological specificity of established lines are not inconsistent with the possibility of accidental cross contamination of strains. Many studies indicate that the cytologic action of antiserum on permanent lines is species-specific (314 to 317). Defendi et al. (318) used separate antisera against HeLa and MCN of human and monkey origin, respectively, to resolve an established line of human amnion cells into two morphologically distinct strains. Additional studies (136, 319, 320, 321) are indicative of the acquisition of common antigens by cells which cross species lines through long-term cultivation. Some of the results could also be accounted for by a common antigen in the form of pleuropneumonialike organisms (136, 321), and others by accidental cross contamination of strains. The extent to which accidental laboratory contamination invalidates certain aspects of comparative cell culture cannot be assessed at the present time. Additional information on the biology and biochemistry of cells will be required before a practical system of determinative cell culture can be established.

Permanent strains provide excellent model systems for studies of some of the factors involved in the transition of cells from in vivo to in vitro conditions. For example, when the environment of an established line is drastically changed by altering the medium or the conditions of culture, the cells are subjected to a situation analogous to that which their predecessors encountered in the primary culture. A number of studies have been conducted to obtain information on the response of populations of established lines to alterations in the environment. Haff & Swim (322) found that RM3-56 cells not only failed to proliferate but gradually degenerated when deprived of

embryo extract. A few survivors were detected as colonies of rapidly proliferating cells in the ratio of about one colony per 106 cells employed initially. The progeny of cells from each clone were capable of sustained growth in the minimal medium. Differential selection applied under conditions in which a large proportion of cells fail to proliferate provides a practical means for examining large populations of cells for the presence of those possessing any of a multitude of distinguishing characteristics. Furthermore, the presence of nonproliferating cells serves the same purpose, in permitting isolated cells to grow, as does the feeder layer described by Puck & Marcus (53). Differential selection has been applied by others to detect the presence of variants in populations of mammalian cells. Swim & Parker (323) isolated several nutritional variants from a strain of U12 which are characterized by differences in their vitamin requirements. Chang (137) described variants of HeLa and of a strain of conjunctiva cells which differ in their abilities to proliferate on various sugars. McQuilkin et al. (152) obtained substrains of L-929 capable of sustained growth in a chemically defined medium. Selawry et al. (324) have described thermotolerant strains. Vogt & Dulbeco (325) selected a variant with increased resistance to poliomyelitis virus from a culture of S3 HeLa. Grosser & Swim (326) have isolated steroid-resistant strains from a cloned U12. It has been a common experience of many investigators (127, 327, 328, 329) to observe, when established lines of cells are placed in a modified medium, a phase of limited growth and extensive necrosis followed by a recovery phase. This sequence is rarely repeated on continued serial cultivation of the strain in the modified medium. Similarly established strains may exhibit new nutritional characteristics on continued serial propagation. For example, the Chang strains of human cells were initially very fastidious with regard to their requirement for certain lots of human serum (286). These strains are currently propagated routinely in media containing serum from any of several species (286, 330). The foregoing observations are consistent with the interpretation that changes in the composition of the medium result in the selection of variants already in the population which are better equipped for growth in the particular medium involved. In addition to those variants isolated by selective procedures, many have been recognized recently on the basis of biological and biochemical criteria. The properties of several biochemical variants (227, 234) were discussed in the section on metabolism. Puck & Fisher (331) isolated the S3 strain of HeLa, which is distinguishable from the parental stock, on the basis of its cloning efficiency in different media. Chang (137) has noted that strains of HeLa from various laboratories differ in their inositol requirements. Sanford and her colleagues (291, 332, 333, 334) have found variations in transplantability and morphology within cloned strains of mouse cells. Puck (312) has stated that the progeny of a clone of HeLa which survived x-irradiation were resistant to the cytopathic action of Newcastle disease virus. Karvotypic variants have also been recognized. Chu & Giles (335) found that colonal

isolates of HeLa were distinguishable from the parental strain and, in most instances, from one another, on the basis of chromosome number and morphology. In contrast to the marked heterogeneity in the parental HeLa, four of the five clonal strains exhibited considerable uniformity in chromosome number. Hsu & Klatt (303) found several uncloned strains to be genetically heterogenous on the basis of a detailed analysis of chromosomal variability. The proportion of mutants in a given population varies with the characteristic involved. In cultures of U12-79 about 1:106 cells can proliferate when inositol is omitted from the medium (198). On the other hand, when choline is omitted the proportion of the cells which survive and proliferate is of the order of 1:104. Sanford et al. (291) and Puck (336) have isolated mutants in the course of examining a limited number of cells by cloning procedures which indicates that certain variants may be present in relatively large numbers in a given population. These observations point up the importance of both selection and cloning procedures in studies of the genetics of mammalian cells. Once isolated and purified by single cell isolation, variants can be propagated serially for many generations in media which do not permit expression of their distinguishing characteristics. Sanford et al. (291, 332) have observed that clonal variants of mouse cells retain their characteristics with respect to transplantability and arginase content for years when grown in their standard medium. Puck (336) has observed that the clonal S3 HeLa retains its distinguishing characteristics after many passages. Similarly, steroid-resistant strains of U12 retain this capacity after many passages in the absence of steroid (198). These data indicate that enzyme induction or some other adaptive process is not responsible for many of the phenotypic changes which have been observed in permanent strains.

The information obtained with established strains supports the conclusion that mammalian cells undergo genetic changes on continued serial propagation. It is not possible at present to distinguish between gene mutation and alternations in chromosomal constitution as potentially responsible mechanisms. In addition, cytoplasmic factors such as those in Paramecium, transducing and transforming agents, and as yet unrecognized mechanisms may be involved. The microbiological aspects of cell culture have evolved to a point where it is now possible to explore these fundamental problems. The use of such a term as mutation is warranted at present only in an operational sense to distinguish divergent properties of the descendents of a single cell. There is no reason to believe that cells during their early life in vitro are more stable genetically than the well-established lines. Mutation and selection could account for the "transformations" which occur when cells from normal tissues are serially propagated. The gradual reduction in the degree of symbiosis which may exist in mixed populations during the initial phases of growth in vitro, and the failure of these cells to achieve the proper genetic combination to permit sustained proliferation in standard media, may be responsible for the frequent lack of success in obtaining permanent strains. When the relationship between mutation and selection is considered, together with the fact that similar media are employed by most investigators, it is perhaps not surprising that permanent strains have many features in common. Increased knowledge of nutrition and its application to the development of different types of media thus becomes a sine qua non in the isolation and maintenance of a broader spectrum of physiological types of cells than is currently available. Permanent lines provide ideal model systems for studies of the basic aspects of genetic control of cells in situ. Such studies are imperative in order to provide a foundation for exploring the apparent mutagenic effects which accompany the normal process of differentiation. and to aid in understanding the mechanism whereby normal cells, on occasion, become malignant. It is in this area that the genetic aspects per se should not be divorced from the environmental factors responsible for phenotypic variability. The potential role of adaptive processes such as induced enzymes, metabolite control of enzyme synthesis and activity, permeability, and possibly unknown mechanisms of biochemical control become of fundamental importance in understanding the developmental physiology of the mammalian cells.

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NEWER ANTIBIOTICS1

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While antibiotic research has had the reputation of being a fast-moving field wherein great developments have occurred with explosive suddenness, in the past several years there has been a marked diminution in the rate with which events of headline proportion have appeared. In the early days of the "antibiotic era" new observations were plentiful, each being greeted some times with more than appropriate enthusiasm. At present, since new developments are viewed against the massive background of the last 18 years of accumulated knowledge, it is not surprising that true progress has become more difficult. Any new antibiotic to be of interest now must carry at least a promise of some features of superiority over all of the impressive array of available chemotherapeutic agents.

It is encouraging that even in this sophisticated field of antibiotic research advances have been made. It will be the purpose of this review to discuss both the progress and the problems which have current importance in relation to the newer antibiotics. Major emphasis will be placed on developments which have become noteworthy from 1956 onward in deference to the two very excellent reviews on antibiotics by Jawetz (1) and Eagle (2) which have already appeared in the *Annual Review of Microbiology*.

To comprehend the trends and directions of the main streams of antibiotic research it is important to realize that the motivation of this research is predominantly economic. Most of the activity, especially in the area of basic discovery and development of new agents, continues to be supported by industry. It is understandable therefore that these funds will be allocated chiefly to areas where success will result in the creation of sizeable markets which exist only where there is a considerable human need coupled with the economic ability to purchase satisfaction. Since altruistic programs aimed at the control of less frequent diseases or diseases of underprivileged populations are scarce, any improved antibiotic therapy for such diseases as brucellosis, subacute bacterial endocarditis, Chagas' disease, blastomycosis, and many other infections, occurs principally as an happenstance extension of the usefulness of an agent developed for some other more profitable purpose. The solution of problems relating to the "unimportant" (unprofitable) diseases will be difficult unless support for research on chemotherapy is derived to a larger extent from nonindustrial sources.

Among the problems of magnitude within the field of antibiotic research, the largest has been generated by the antibiotics themselves, namely, that of infections caused by "antibiotic-resistant" staphylococci. A hospital nursery epidemic caused by penicillin-resistant staphylococci was reported by

¹ With a few exceptions, the survey of the literature pertaining to this review was concluded in December, 1958.

Barber et al. (3) as early as 1949 and it was not long before epidemics were described among newborn infants in hospital nurseries over almost all areas of the world. Although these infections were not limited to newborn infants and their mothers, the spectacular and devastating effects of staphylococcal infection in the nursery has tended to focus major attention upon these epidemics. Now, however, the epidemiology of infections caused by antibiotic-resistant staphylococci has been revealed in its broader aspects and the seriousness of the situation is becoming more widely recognized. Briefly stated, certain strains of staphylococci, usually belonging to a limited number of phage types including phage type 80/81 (4), have simultaneously displayed the presumably independent properties of high virulence and resistance to several of the most commonly used antibiotics. These strains are resistant to penicillin, streptomycin, and the tetracyclines; many are also resistant to erythromycin. Whereas staphylococcal disease in hospitals was originally considered to be an epidemic situation, it is now recognized as a widespread endemic problem with the constant threat of epidemic resurgence. Wysham & Kirby (5), and Felton (6), have reported mortality rates of 21 per cent and 14 per cent, respectively, among hospitalized patients developing antibiotic-resistant staphylococcal infections. Normal, otherwise healthy persons, easily become carriers of these organisms and there is now evidence that they have been disseminated into the general population (7, 8). Although infections among infants and debilitated persons are the most serious, the presumably healthy adult may also become infected, though usually to a milder degree.

When one considers the frequency, severity, and mortality of antibiotic resistant staphylococcal infection, it is not surprising that research directed toward the control of these organisms is by far the largest effort in the field of antibiotics at the present time. Most of the newer antibiotics currently under investigation and development are agents offering some hope of

effectiveness against these organisms.

Another area of active antibiotic research concerns agents of possible use in the treatment of tuberculosis. Though tuberculosis mortality decreased sharply with the use of streptomycin, p-aminosalicylic acid (PAS), and isonicotinyl hydrazide, present mortality rates still leave much to be desired. In addition, the facility with which tubercle bacilli develop resistance even when exposed simultaneously to several agents makes it obvious that tuberculosis therapy is far from satisfactory. Current therapy is plainly of borderline effectiveness compared to that for most other infectious diseases. Therefore, it is logical that hope of developing really effective therapy has persisted in many laboratories. Fitzpatrick (9) may have signalled a changing approach to the search for new therapeutic agents by comparing the activity and toxicity of drugs on the basis of an ultrashort treatment regime. There seems to be little real reason to assume that a drug must be able to be administered for long periods to be effective against Mycobacterium tuberculosis.

The dermatophytic mycoses collectively have probably the highest incidence of any infectious condition, yet they are generally sufficiently mild that they can be classified as troublesome rather than serious. Hundreds of "remedies" already exist for these conditions. This is clear evidence of the relative lack of effectiveness of most of them. Nevertheless, any new and truly effective agent must compete for recognition in this already-crowded field and it is likely that it would be both more costly and more toxic. The magnitude of the human and animal need in this field makes it attractive, but the problem of exploiting a truly effective agent has been a dominating hindrance. Therefore, few if any laboratories have committed their efforts strongly to a search for antifungal therapy but several are devoting a small but continuing effort in this direction. The importance of antifungal agents in agriculture and industry may provide therapeutic agents as "byproducts" of research in these areas. Indeed, one such agent will be discussed in this review.

Neoplastic disease now is replacing infectious disease as a major health problem. While heretofore the principal targets for antibiotic action have been the metabolic functions of disease-producing microorganisms, recently many investigators have set their sights on the metabolism of neoplastic cells. The progress made in the *in vitro* cultivation of tissue cells now provides study techniques almost as convenient as those for screening antibiotics against bacteria. As a result, broad searches for "antitumor" activity have become practical, and the products of microbial cells are promising materials for study. Many laboratories have on hand large numbers of "toxic" antibiotics of varying degrees of purity which are immediately available for experimentation.

The foregoing discussion has outlined some of the areas of particular activity in the field of antibiotic research. Of course, there are other areas of interest and certain of these will be mentioned. However, for whatever convenience it may offer, the individual agents will be grouped for discussion according to what currently appears to be their areas of principal importance.

THE ANTISTAPHYLOCOCCAL ANTIBIOTICS

Novobiocin was the first antibiotic to be introduced, chiefly because of its activity against staphylococci resistant to other antibiotics. Very soon after its description by Wallick et al. (10) and Smith et al. (11) (independently this agent was shown to be active against staphylococci, pneumococci, and various other Gram-positive bacteria both in vitro and in experimental infections in mice (12 to 15). Although Gram-negative organisms were much more resistant, certain strains of Proleus were sufficiently sensitive to offer some hope of therapeutic response (13). Clinical investigation confirmed the activity of novobiocin against staphylococcal, pneumococcal, and strept-coccal infections (16 to 19). The response of Proleus infections was variable (20, 21) but still of interest because of the lack of other more satisfactory therapy. Novobiocin does not show cross-resistance with any other clini-

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cally used antibiotic, and studies of large numbers of staphylococci have shown almost complete sensitivity of these organisms to this drug (22). Although staphylococci can develop increased resistance to novobiocin within 10 to 12 serial transfers (22), and Nichols & Finland (20) isolated staphylococci of increased resistance from certain patients during novobiocin therapy, it does not seem that this has yet become a serious therapeutic problem despite the very wide use of this drug. Doses of 1 to 2 gm. per day, which are commonly employed, produce plasma concentrations that are quite high and prolonged compared to those of other antibiotics (23, 24). However, novobiocin is highly bound to plasma protein (14, 25), so the concentration available for antibacterial activity is somewhat lower than plasma concentrations would imply. Novobiocin does not enter the spinal fluid readily (25). It causes little significant primary toxicity when used at its recommended dosage, but rashes will occur in about 10 per cent of cases, particularly among those who are treated for a week or more. These subside quickly when the drug is withdrawn. Although penicillin and the broad spectrum antibiotics continue to be preferred for treatment of nonstaphylococcal infections, the combined use of novobiocin with penicillin or a tetracycline antibiotic is common in situations where the etiology of an infection has not been determined.

Oleandomycin is another antibiotic that has received attention as a possible agent for oral use against antibiotic-resistant staphylococci. It was described by Sobin et al. (26) in 1955 as an agent predominantly active against Gram-positive bacteria. Subsequently, English et al. (27) described the properties of a combination of $\frac{2}{3}$ tetracycline and $\frac{1}{3}$ oleandomycin, which was reported to have an action sufficiently enhanced to render even tetracycline-resistant staphylococci sensitive to the mixture. While Garrod (28) has not found a significant synergistic interaction between these two antibiotics, the major question is the practical significance of the relationship between oleandomycin and erythromycin. Although laboratory-developed cross resistance is complete, English et al. (29) have reported that many naturally occurring erythromycin-resistant organisms were still sensitive to oleandomycin. Koch & Lepley (30) and Rantz et al. (31) found that among naturally developed erythromycin-resistant staphylococci, cross resistance to oleandomycin is common but does not occur invariably. Garrod (28) has demonstrated that oleandomycin is considerably less active than erythromycin. Considering the increasing frequency of staphylococci resistant to erythromycin, the use of oleandomycin either alone or in combination with tetracycline seems to require rather precise knowledge of the susceptibility of the patient's infecting organism. Recently, the triacetyl derivative of oleandomycin has been made available (32). This compound is more stable to gastric acidity and deacetylates at a rate which approximately doubles the oral absorption of active oleandomycin (33). While this improves its activity in comparison to erythromycin, it does not affect materially the erythromycin-oleandomycin resistance problem. However, oleandomycin and its triacetyl derivative are relatively nontoxic at usual dosages and are effective in the treatment of susceptible infections (34).

A second new antibiotic of the erythromycin group is spiramycin (35). Although cross resistance with erythromycin is not complete in either laboratory strains or naturally resistant strains, Garrod (28) found 31 of 45 naturally erythromycin-resistant staphylococcus strains to require eight or more μ g. of spiramycin for inhibition. Since plasma concentrations attained during treatment range around two μ g./ml. of spiramycin (36), cross resistance appears to be sufficiently great to pose a therapeutic problem. In vitro data suggest that this agent may be considerably more effective, against pneumococci and Streptococcus pyogenes than against staphylococci.

Although activity by the oral route has been considered almost a necessity for the acceptance of new chemotherapeutic agents, the seriousness of the problem of antibiotic-resistant staphylococcal infection has made this requirement less important. Recently several new antibiotics have been introduced that are poorly absorbed orally, but when injected seem to have significant therapeutic activity. One of these is ristocetin which, at present, must be given intravenously. This agent contains two components, and both are active against Gram-positive bacteria, notably staphylococci, pneumococci, and streptococci. The antistreptococcal activity is of particular interest since it extends to Streptococcus faecalis and the enterococci that are serious problems in bacterial endocarditis. Cross resistance has not been found to occur with any other clinically useful antibiotic and resistance development was slow. Natural resistance was not found in a study of more than 400 streptococcal, pneumococcal, and staphylococcal cultures (37, 38). Ristocetin is actively bactericidal for streptococci and penumococci near its inhibitory level and, in higher concentrations, produces this action against staphylococci and enterococci (39). Romansky et al. (40, 41) have reported the successful use of this agent in the treatment of pneumococcal pneumonia and staphylococcal and enterococcal endocarditis. Taylor et al. (42) have had success in its use in staphylococcal pneumonia when used alone or in combination with other antibiotics. Unfortunately, a temporary leukopenia has occurred in some patients at high dosage. Because of this, and the necessity for intravenous administration, ristocetin is an agent having its major use in the seriously ill hospitalized patient. Here it appears to have lifesaving possibilities.

Another antibiotic very similar to ristocetin in its general properties is vancomycin (43). Although chemically different and not cross resistant with ristocetin or any of the other commonly used antibiotics, it must be administered intravenously and is active almost entirely against Gram-positive organisms (44). Pneumococci, clostridia, and streptococci of the viridans, mitis, and faecalis groups are among the organisms which are relatively sensitive to vancomycin (45), but its antistaphylococcal activity has received major clinical attention (46). Several studies have emphasized the fact that staphylococci are very slow to develop resistance in vitro (44), and

Kirby & Divelbiss (46) have found little or no change in sensitivity with staphylococci isolated from patients who have been on therapy for several days. Two μ g./ml. has usually been adequate to inhibit the *in vitro* growth of most Gram-positive organisms and intravenous administration of 1 gm. twice a day produces serum concentrations that are considerably above this value (46, 47). It is excreted by the kidney at a rate comparable to that for tetracycline (46). Although no definitive information on plasma binding seems to be available, the relative lack of inactivation by serum indicates that vancomycin is not highly bound to plasma proteins (46). Penetration into most body fluids and exudates is quite good, but vancomycin does not appear significantly in the spinal fluid (47). It has been used with success in the treatment of serious systemic staphylococcal infections, including endocarditis (46 to 49), and Geraci *et al.* (47) have been impressed with its action when given orally to patients having staphylococcal ileocolitis. Here its insignificant oral absorption may be a virtue.

At this time, clinical experience with vancomycin has not accumulated sufficiently to provide evaluation of the true nature and frequency of untoward reactions. However, "drug fevers" which may or may not have been caused by contaminating pyrogenic impurities, have occurred with moderate frequency (46). Rashes have also been reported (47), but of greater seriousness is the report by Geraci et al. (47) concerning the occurrence of tinnitus and impaired auditory acuity occurring in some patients having high (>90 μ g./ml.) plasma concentrations. Like ristocetin, the need for intravenous administration and the possibilities of relatively serious side reactions makes vancomycin an antibiotic that should finds its major usefulness in the seriously ill, hospitalized patients.

Unfortunately for the reputations of both ristocetin and vancomycin, they will probably be used in many patients after all other therapies have failed. This results in the employment of maximum dosage in patients who have the least likelihood of recovery. Under these circumstances, apparent therapeutic effectiveness is minimized and apparent toxicity is magnified. This should be recognized in evaluating reports of the use of these antibiotics.

Japan has contributed a new antibiotic which has recently been receiving intensive evaluation. This agent, described by Umezawa et al. (50), has been named kanamycin and appears to be somewhat similar to neomycin both in its biological properties (51, 52) and in some aspects of its chemistry (53). Most of the available information about kanamycin has been summarized in the report of a symposium published recently (54). This antibiotic is known to consist of two components (A and B). Although it appears to be slightly less active than neomycin against several species of bacteria, it is a broad spectrum agent offering significant action against staphylococci, coliforms, Shigella, and Salmonella (51, 55). It also is inhibitory for Mycobacterium tuberculosis (56). Kanamycin is not absorbed from the gastro-intestinal tract, but it is readily absorbed after intramuscular injection and

seems to distribute into tissues quite well. Tisch, Huftalen & Dickison (57) using dogs, found that kanamycin entered the spinal fluid in significant concentration. Although this antibiotic is cross resistant with neomycin, it does not cross with streptomycin or the other antibiotics outside of the neomycin group (55). Kanamycin has been employed successfully in the parenteral treatment of staphylococcal infections (58, 59) and in coliform and proteus urinary tract infections (60). Some other miscellaneous infections have also responded to treatment (59). Its action on tuberculosis appears to be definite (61), but there seem to be no indications of any advantage over existing methods of treatment. Unfortunately, kanamycin has shown evidences of some renal irritation and definite signs of toxicity for the auditory branch of the 8th cranial nerve (62). Although nephrotoxicity is usually reversible at normal dosage, decreases in auditory acuity appear to be disturbingly common and permanent (63). The incidence of 8th cranial nerve involvement is related to the dosage level and duration of treatment. Relatively large doses have been given for a few days without difficulty. There appears to be little question that kanamycin is a useful antibiotic in the treatment of staphylococcal infections which are resistant to the "older" antibiotics; however, patients receiving this agent should be under continuous observation, and the duration of the therapy should be kept to a minimum.

Of the many antibiotics described as having antistaphylococcal activity, those so far mentioned have been agents possessing a confirmed degree of therapeutic utility. Streptogramin (64), staphylomycin (65), PA 114 (66), and E 129 (67) have not yet reached this stage of development. Since these agents appear to be somewhat related on the basis of their biological properties, and De Somer & Van de Voorde (68) have reported staphylomycin to be effective in studies involving fifty patients, there is at least the hope that some or all of these may become useful antibiotics. All of these substances are active predominantly against Gram-positive organisms including staphylococci, pneumococci, and streptococci. All are mixtures of two or more substances which appear to interact synergistically. Thus, there is now streptogramin fractions I, II, and III (69), staphylomycin fractions MI, MII, and S (70), PA 114 A and B (66), and E 129 and E 129B (67). The interrelationships among these agents and their fractions are still under investigation, but cross resistance has been found by De Somer & Van Dijck (65) between staphylomycin and streptogramin, and Van Dijck et al. (71) have also found both cross resistance and cross synergism between certain of the fractions of staphylomycin and PA 114. Garrod & Waterworth (67) found E 129 to be partially cross resistant with erythromycin and English et al. (72) reported PA 114 resistance in staphylococci made resistant to erythromycin. Streptogramin (73), PA 114 (72), and staphylomycin (65) are protective against staphylococcal, streptococcal, and pneumococcal infections in mice when given orally as well as parenterally. Unlike erythromycin

and spiramycin, their antipneumococcal activity appears to be relatively inferior. Streptogramin and staphylomycin are the most similar in their biological and distributive properties. Both agents disappear from the blood soon after injection and do not appear in the urine in significant quantities. Despite this, De Somer & Van de Voorde (68) demonstrated a protective effect in mice for staphylomycin given 6 hr. before infecting the animals. They have suggested an absorption to and progressive release from tissues. This may explain the unusually good effect found by Verwey et al. (73) in topical therapy of infected experimental wounds with streptogramin, and by De Somer & Van de Voorde (68) in topical staphylomycin treatment of local infections in patients. Staphylomycin was effective when given orally in four doses at a level of 1.5 to 2.0 gm./day to patients with various staphylococcal infections, and no significant toxicity was noted. Considering the other antistaphylococcal antibiotics available for parenteral use and the mediocre results so far obtained with topical therapy, the effectiveness of staphylomycin and streptogramin by this route may be the most interesting property of these antibiotics.

It is apparent that we are not without newer antibiotics which offer reasonable hope of therapy for infections that are resistant to the "older" antibiotics. However, none of these agents is free from serious disadvantages which dictate discrimination in its use. The effectiveness of these newer substances depends to a major extent upon the early recognition of resistant infections and the quick and accurate selection of the proper agent for use. Close coordination between the clinic and the laboratory is becoming even more essential than ever.

ANTITUBERCULOSIS ANTIBIOTICS

During the period covered by this review the only new agent to receive extensive study in human tuberculosis has been cycloserine, an antibiotic described by Harned et al. (74). The evaluation of antituberculosis agents is long and complex, but there appears to be an increasing interest in cycloserine. Though of very low in vitro activity (75), Epstein et al. (76) found it impressive when used alone at 1.0 to 1.5 gm./day, and numerous other studies have confirmed its clinical effectiveness (77 to 81). Central nervous system toxicity is a definite property of cyloserine (80, 81, 82) and its ultimate utility will depend upon the circumvention of these side effects. This has been attempted by reducing the dosage (78), employing reduced dosage with isonicotinic hydrazide (INH) (83) or viomycin (84), and co-administering pyridoxine (79) or anticonvulsant drugs (85). The data concerning the facility with which M. tuberculosis becomes resistant to cycloserine are somewhat conflicting (78, 83), but at least resistance seems to be no more of a problem than with INH. Cycloserine is well absorbed orally and is quite rapidly excreted (75). Pleural fluid, ascitic fluid, and spinal fluid concentrations are approximately equivalent to plasma concentrations (86). This excellent and unique penetration into tissue may be a major factor in explaining the unexpectedly favorable results that have been obtained in chronic tuberculosis where avascular areas of infection are a dominating problem. Although Herrold *et al.* (87) have found cycloserine to be effective in the treatment of various urinary tract infections, there seems to be little interest in this agent except as an antituberculosis drug.

The activity of kanamycin in the therapy of tuberculosis infections has been mentioned previously (see page 183). It is too early to make any sound evaluation of this agent, but its tendency to produce irreversible damage to the auditory branch of the 8th cranial nerve is certainly a hindrance to its use in tuberculosis for anything except immediate protection during and following surgical intervention. The discovery of a detoxicant for kanamycin would alter the outlook for this antibiotic considerably.

ANTIFUNGAL ANTIBIOTICS

Antibiotics having an *in vitro* activity against pathogenic fungi are relatively common, but few have ever been reported to have *in vivo* activity against the fungi causing systemic infection. Although several such agents have been described recently including candidin (88, 89), eulicin (89, 90) and amphotericin A and B (91, 92, 93), clinical studies have been reported only with amphotericin B. This must be given intravenously for systemic absorption but it has been reported to have activity in the treatment of histoplasmosis (94, 95), blastomycosis (95), and cryptococcosis (96). More extensive trials in histoplasmosis and coccidiodomycosis are under way (97). The degree of therapeutic activity is certainly borderline and long-continued treatment seems necessary. As in tuberculosis, the evaluation of therapy for treatment of the systemic mycoses requires a long time, but the increasing recognition of the systemic mycoses lends importance to the problem.

The search for antibiotics that might be effective against the dermatomycoses has been handicapped by the lack of convenient experimental infections in animals which are not self-limiting. Recently, Williams et al. (98) and Blank (99) have reported that griseofulvin was active in the treatment of certain dermatomycoses in humans when administered orally. These are the first reports of an orally active agent for this purpose. Successfully treated infections have included those involving hair, skin, and nails and have been caused by various species of Microsporon and Trichophyton, but infections caused by Blastomyces and Candida species have not responded (99). Griseofulvin seems to be quite nontoxic since doses of 1 to 2 gm./day have been given for periods up to three months. No toxicity has yet been seen in humans, but animals on very high dosage have shown depression of the hemopoietic system.

Griseofulvin is an old antibiotic (100) that was studied first as an agricultural fungicide and has only recently been tried in animals (101, 102). It is produced by several penicillium molds and Grove et al. (103) proposed

a new chemical structure in 1952. With the impetus of the interest aroused by its recent oral activity in humans, attempts to confirm its structure and reports on related compounds should appear soon.

ANTITUMOR ANTIBIOTICS

The upsurge of activity in the cancer-chemotherapy field has had repercussions in the antibiotic area, and broad screening programs are in progress which have as their objective the discovery of agents selectively toxic for neoplastic tissue. While nothing of proven therapeutic value has yet been derived from these studies, the reports on actinomycin D (104), azaserine (105), 6-diazo-5-oxo-L-norlencine (DON) (106, 107) and mitomycins (108) are illustrative of the activity in this field. Definite inhibition of tumor growth has been obtained in experimental animals by orally active substances (105, 107). The antimetabolite approach to tumor inhibition is thoroughly rooted in this field and has been pursued with great skill. It will be interesting to see whether this or the broad screening of unknown "antibiotics" will be more productive.

ANTIVIRAL ANTIBIOTICS

It would be a serious omission to conclude a review of recent antibiotics without at least a mention of the activity in the area of antiviral agents. The state of this field has recently been reviewed by Cutting & Furst (109) who have summarized the situation as follows: "Since 1949 we have accumulated without any special effort except observation, more than 1100 references to virus chemotherapy. None has been the crucial paper; that is, none has reported a really satisfactory agent, although at the laboratory level a number of substances have had minimal effects... Virus chemotherapy is a considerably tilled field, but with little harvest." There seems to be no basis for disagreement with this observation. However, the fascination of the slot machine. Pay-offs are plentiful enough to maintain the gambling fever, so—if someone will supply the coins, the game will go on and on, and on. Who knows? The next jackpot may be a "cure for cancer" or an effective antiviral agent.

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PLANT DISEASE RESISTANCE1

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"The nature and variability of resistance is one of the most challenging, fascinating, and important fields of plant pathology, and it merits thorough research" [Stakman & Harrar (98)]. It is with this stimulating thought in mind that a brief review of the facts, interpretations, and theories of plant disease resistance is undertaken. This review is intended to bring together some of the varied and sometimes seemingly conflicting conclusions in an attempt to fit the existing bits of knowledge into a more unified picture of the nature of disease resistance in plants. Emphasis will be placed on the physiological aspects of the host-parasite relations, and especially those of the host which lead to inhibition of parasitism and of pathogenicity by fungi and bacteria.

After surveying the literature on parasitism and resistance, one is impressed by how much has been written about so few well-established facts. Theories have been expressed freely, but much of the research has not been carried on extensively or intensively enough to obtain all of the facts necessary for firmly-established conclusions. Space does not permit the inclusion of all of the many pertinent references on this and related subjects, but additional references may be found in recent reviews of other phases of parasitism and resistance [Allen (2); Brown (15); Christensen & DeVay (22); Dimond (23); Eide (25); Farkas (26); Kern (57); Walker & Stahmann (113); Yarwood (116)].

Resistance to a given plant disease is a stable character and has its basis in the genetic composition of the host plant, but the expression of the genes may be modified by nutritional and environmental factors. Resistance in the higher plant to a specific pathogen may be attributable to a single dominant gene, to a pair of recessive genes, or to multiple genes acting collectively. Recent genetic studies have revealed the complex nature of reactions between varieties of a given host and pathogenic races of a given fungus [Black (10); Flor (31)]. Genes for resistance in host varieties are believed to be matched by a corresponding set of genes for pathogenicity of the races. Resistance results only when specific race-variety combinations occur. Since genes are known to control the production of certain enzyme systems, the physiological activities of both host and parasite are of prime importance in determining the degree of resistance or susceptibility.

The first response of a potential host is determined largely by the initial acts or secretions of the parasite during penetration and invasion. Allen (1) pointed out that the symptoms of a parasitic disease are largely the result of

 $^{^{\}rm 1}$ The survey of literature pertaining to this review was concluded in September, 1958.

an interchange of chemicals between host and parasite. For this reason, a review of the subject of plant disease resistance must include some discussion of the activities of plant parasites.

PARASITISM

The basic principle of parasitism is one of nutrition involving two living organisms of different species, one of which is the donor and the other the receiver of nutritional substances. Some microorganisms are known only as parasites in or on living plant tissue. The conditions under which these parasites may be able to grow in the absence of the living host are not known. These organisms have been called obligate parasites and, since they have not been successfully cultured on any other medium, are believed to require living tissue as a substrate. However, the number of species in this group is becoming smaller year by year as knowledge of fungus physiology is extended. Thus, the term "obligate parasite" is frequently inappropriately used. It reflects only the inadequate knowledge of parasite nutrition and does not describe a basic mode of parasitism, even though it is sometimes used with this implication.

The terms proposed by Gäumann (39, 40), "biotrophic parasites" and "necrotrophic parasites," seem to this author to describe more accurately the two basic methods of obtaining nutrients under natural host-parasite relations. The biotrophic parasites would include those which obtain nutrients from living cells of the host, regardless of whether they can grow on artificial media. The nectrotrophic parasites would include those which utilize nutrients from dead host cells, usually those killed by the parasite itself.

The filamentous biotrophic parasites obtain nutrients by sending haustoria into living cells, or by making close contact with living cells without the formation of haustoria. Gäumann (39) considered the haustorium as a growth form determined by the defensive actions of the host cells and the counter reactions of the invading hyphae.

Entrance of parasites into a host may be gained by direct penetration, through natural openings, or through wounds. Walker & Stahmann (113) have pointed out that in some fungi, particularly the rusts, the mode of penetration is genetically controlled by the parasite. For example, the monocaryotic germ tubes of *Puccinia graminis* penetrate the epidermis directly through the cells, while the dicaryotic germ tubes of aeciospores and uredospores penetrate through stomata. Cutinized walls of epidermal cells are penetrated mainly by mechanical pressure, while penetration of cellulose walls may be aided by enzymes. Wounds in host tissue usually result in the death of some of the surrounding cells and hence do not favor invasion by biotrophic parasites. On the other hand, sap secretions from wounds frequently are excellent media for germination and early growth of necrotrophic parasites.

Some biotrophic parasites make close contact with their hosts without

penetrating the host cells during the parasitic phase. Venturia inaequalis penetrates only the cuticle under which it forms a compact layer of hyphae and absorbs nutrients from the epidermal cells. Other good examples of this type are found among the mycoparasites [a term used to designate fungi parasitic on other fungi by Butler (18) and by Christensen & DeVay (22)]. Rhizoctonia solani coils around hyphae of certain hosts without penetration [Butler (18)]. Calacrisoproim parasiticum contacts it host hyphae at specific points without penetration and establishes a balanced nutritional relationship with little harm to the host [Barnett & Lilly (6)].

Following penetration of the host, the survival of the parasite is determined by a series of interactions between the two organisms. The rot-inducing pathogens produce specific pectolytic enzymes that break down the pectic cell wall material and cause death of the host cells [Brown (15); Fushtey (32)]. Wilt pathogens are believed to bring about wilting by means of toxins, polysaccharides, or pectolytic enzymes which act on the pectic materials of the vessels [Dimond (23); Gothoskar et al. (45)]. Some pathogens are known to produce specific toxins, such as fusaric acid, lycomarasmin, alternaric acid, victorin, and the wild fire toxin [Braun (12); Gäumann (41, 42); Krupka (62)]. Defense reactions of the host against toxins have been discussed by Bloch (9). Initial secretions by biotrophic parasites usually cause only a mild reaction in a susceptible host.

Many investigators have reported respiratory changes in diseased plants. Allen (1) suggests that the metabolism of normal host cells is regulated to prevent undue expenditure of reserves and yet maintain their own life processes and growth. This regulatory mechanism is destroyed by the invading parasite, and the food reserves and intermediate products become available to the parasite.

The success of an organism as a parasite is largely a result of its aggressiveness, a term which is used to describe the ability to penetrate, overcome host resistance, absorb its needed nutrients, and spread through host tissue [Gäumann (40)]. Aggressiveness is fundamentally different from pathogenicity, the ability to incite a disease. Strong aggressiveness in a given organism may be combined with weak pathogenicity or weak aggressiveness with high pathogenicity.

RESISTANCE

In this review, resistance is considered to be the result of all of the factors which tend to prevent or reduce the aggressive or pathogenic activities of the parasite after contact with a potential host has been made. In some cases only one factor is involved, while in others resistance may be the result of several factors acting either simultaneously and collectively, or individually. The complex interaction of several factors renders understanding of the nature of some host-parasite relationships difficult. For convenience, the known resistance factors will be discussed in two groups, those which act to prevent penetration of the host and those whose action is involved after penetration.

ANTIPENETRATION FACTORS

These factors may be either mechanical or chemical. They represent inherent characters of the healthy host and, for the most part, may be considered as passive. There is little or no response of the host to the act of penetration alone, unless secretions of the parasite are involved. Resistance of this type frequently increases with age of the host tissue. Brown (14) has pointed out that the protection afforded to a plant by its outer layers of cuticle or bark is of great importance. Many plants are immune to attack by certain fungi only as long as the protective layer is unbroken.

Mechanical resistance.—In the higher plants, mechanical resistance to penetration is attributable mainly to a suberized layer of cork cells or to cutinized epidermal cells. The cuticle on aerial parts not only reduces loss of water but also reduces excretion of solutions which might provide nutrients for the external growth of certain parasites. Some necrotrophic parasites are apparently unable to penetrate the unbroken cuticle, unless they are able to

make some saprophytic growth on the surface [Brown (15)].

Among the mycoparasites, infection hyphae of Rhizoctonia solani were often walled off by a sheathlike covering in Rhizopus nigricans, Phycomyces blakesleeanus, and Mucor recurvus [Butler (18)]. Penetration of chlamydospores of species of Endogone by an unidentified fungus parasite occurred through slender canals in the thick spore wall. Sometimes the invading hyphae were surrounded by a sheath of wall material growing inward. Frequently the invading hyphae failed to break through the sheath [Godfrey (44)]. In both cases, the formation of the sheath was interpreted as a reaction by the host serving to restrict growth of the invading hyphae. In a similar way, the age of hyphae of some species of Mucorales is an important factor limiting penetration by haustoria of Piptocephalis virginiana [Berry (7)].

Chemical resistance against penetration.—One of the best known examples of a chemical barrier to penetration is illustrated by the resistance of colored onions to Colletotrichum circinans. Since this mechanism has been reviewed recently by Walker & Stahmann (113), only the essential facts will be presented here. The colored, dead outer scales of red and yellow onions were found to resist penetration by C. circinans, but if the colored scales were removed these bulbs were attacked as readily as were white varieties. Phenolic compounds, protocatechuic acid, and catechol, were isolated from the dry, colored outer scales [Link et al. (66); Link & Walker (67)]. These compounds in a water-soluble form are present in the dry outer scales and diffuse out into the infection drop of moisture and prevent germination of spores of C. circinans. Similar extracts of colored onion scales were found to inhibit Diplodia natalensis and Botrytis allii [Ramsey et al. (75); Walker (110)]. Since these phenolic compounds were not found in the living cells of colored onions, it was concluded that nontoxic precursors were present in living cells and the toxic compounds were formed as the cells die.

A different type of chemical resistance of the gram plant to penetration

by Mycosphaerella rabiei is reported by Hafiz (50). He concluded that the greater number of glandular epidermal hairs found on the resistant plants than on susceptible plants resulted in more extensive secretions of malic acid which inhibited spore germination or germ tube development.

It should be pointed out that chemical or physiological resistance is mainly active after penetration by the parasite. Further discussion of this

type will be given later.

RESISTANCE AFTER PENETRATION

The period immediately following penetration into a potential host is critical for the parasite. During this period (infection and invasion) the successful parasite establishes a nutritional relationship with the host and spreads through its tissues. It has been pointed out that during the host-parasite relationship there is an exchange of chemicals between the two organisms [Allen (1); Brown (15)]. It appears that the first secretions by the parasite serve as stimuli which start a chain of events which ultimately lead to successful parasitism on the part of the parasite or to successful resistance on the part of the host. Some resistance factors act primarily against infection and the initial phases of invasion, whereas others may reduce the extent of invasion and modify the severity of the disease.

Structural responses to invasion.—Response of a host to an invading parasite may be either structural or chemical. While structural changes may be of lesser importance, they occur frequently and in some cases undoubtedly contribute materially to the resistance of the host. Some leaf spot organisms, such as Coryneum beijerinkii, produce a zone of dead tissue around the infection and stimulate the formation of suberized cells at the edge [Samuel (80)]. The infection may be eliminated when the dead tissue shrinks and falls out. Layers of cork cells in roots are said to slow down the invasion of Armillaria mellea [Thomas (101)]. Stereum purpureum stimulates the production in vessels of resistant plum varieties of gums which check further growth of the fungus [Brooks (13)].

Sheathlike thickenings of the host cell walls containing suberin, lignin, or other similar substances have been reported in several host-parasite relations [Burges (16); Fellows (27); Young (117)]. Penetration of both epidermis and cortical cells of the hop root by species of *Verticillium* was slowed down by lignification of the cell walls, and invasion into the vascular system was reduced by suberization of the cells of the endodermis [Talboys (99)]. Sheathlike wall thickenings around the invading hyphae were also common in the hop root. In susceptible hosts, the sheaths were frequently perforated by a virulent strain of the pathogen but seldom by a mild strain. In resistant hosts the sheaths were rarely perforated, even by the virulent strain. A variety of cucumber resistant to *Cladosporium cucumerinum* showed that invasion resulted in thickening of the parenchyma cell walls and in cell necrosis, confining the infection to a limited area [Pierson & Walker (73)]. Similarly,

the response of resistant cucumber tissue to invasion by *Colletotrichum lagenarium* was accompanied by thickening of host cell walls and slow destruction of host cell contents [Busch & Walker (17)].

The size of lesions produced by *Colletotrichum lindemuthianum* on susceptible bean plants was inversely proportional to the age of the infected tissue [Leach (65)]. Thus, susceptible tissue became resistant with age. Other physiological responses may occur at the same time and may actually exert greater effects against the invading pathogen than do the structural changes.

Absence or unavailability of nutrients required by parasite.—Resistance of this type may result from (a) the absence of required nutrient in the host; (b) an inadequate supply of a required nutrient at the infection site; or (c) the inability of the parasite to obtain the nutrient. The lack of specific nutrients as a basis for resistance would probably apply principally to the biotrophic parasites, since some of them are known to be more exacting in their nutritional requirements.

Leach (64, 65) studied the resistance of wheat to Puccinia graminis and of bean to Collectotrichum lindemuthianum and reached similar conclusions in both investigations. He proposed the theory that both of these parasites required from their respective living hosts specific nutrients furnished only by susceptible hosts. On the basis of histological studies, it was concluded that the mycelium of the parasite entered the cells of a resistant host and, in the absence of required nutrients, it soon died from starvation, and its autolytic products then killed the surrounding host cells. C. lindemuthianum grew equally well on expressed cell sap of both resistant and susceptible hosts. Wellensiek (114) concluded that Leach's theory also explained resistance and susceptibility of corn to Puccinia sorghi. This hypothesis is also accepted by Chester (21) to explain resistance to other rusts. If this theory is accepted, physiologic specialization in the biotrophic parasites would be explained on the basis of the presence or availability of many specific nutrients in different host varieties. While it is extremely difficult to test the validity of this theory using parasites which can be cultured only on living hosts, it should be pointed out that Colletotrichum lindemuthianum is able to grow on a synthetic medium, without additions from a susceptible bean plant.

Leach (64) also suggested the possibility of metabolites being present in different hosts in different stereoisomeric forms and that the ability of the parasite to use only certain forms may be the basis of resistance. The possibility of isomeric transformation of metabolites in tissues of the host was suggested by Sempio (86), who reported that experiments were underway to determine the possibility of stimulating plants toward this type of isomeric resistance. The lack of direct supporting evidence is one of the principal arguments against this theory. Perhaps the use of paper chromatography and other modern tools in the hands of trained biochemists will, in the future, throw more light on its validity. For this purpose it would seem that host-parasite combinations shown to have a gene-for-gene relationship for resistance and pathogenicity, would be a wise choice.

One of the best examples of a biotrophic parasite requiring for its growth a specific vitaminlike nutrient furnished by living host cells is that of Calcarisporium parasiticum [Barnett & Lilly (6)]. This parasite makes good growth on several fungi, including Physalospora obtusa and some related species, but it fails to parasitize Physalospora rhodina and certain other species or to grow on culture media without a host. The parasite makes contact with P. obtusa (susceptible) and P. rhodina (immune) alike at specific points but penetrates neither. When a small amount of a water extract of finely chopped host mycelium was added to a complete agar medium containing yeast extract or glutamic acid, C. parasiticum made slow continuous growth. The extract of P. rhodina was equally as effective in promoting growth as that of P. obtusa. The required extract did not replace either the carbon or the nitrogen source in the medium. The growth factor is effective at high dilutions, within the active range of some vitamins, and is heat stable. It is held within the living cells of the host and is not secreted into the culture medium. The chemical identity of this growth factor has not been determined

The growth factor required by Calcarisporium parasiticum is not present in all of the fungi tested and immunity of many fungi to this parasite may be caused in part by the absence of the specific nutrient. However, immunity of P. rhodina must result from an entirely different factor, since the mycelium of this species was shown to contain the required growth factor. In this case it appears that the parasite is able to absorb the required nutrient from certain species but not from other species of fungi. This may be attributed to differences in local permeability changes of the host membranes initiated by a secretion from the parasite.

The necrotrophic parasites are generally considered to be less specific in their nutrient requirements and usually can be cultivated on a variety of media. Allen (1) has pointed out that the factors determining pathogenicity may be different from those affecting growth of a parasite. It is equally true that resistance may be caused by factors other than those which inhibit growth. For example, Brown (15) showed that the production of pectolytic enzymes responsible for rotting host tissue does not occur under all conditions that permit growth of the pathogen. Thus, according to our present knowledge, it appears unwise to draw too many conclusions regarding the role of specific nutrients in determining resistance or susceptibility. While the absence of a specific nutrient in a potential host may result in resistance to a parasite deficient for this nutrient, the converse is not true. It cannot be concluded generally that the presence of a required nutrient is the basis for susceptibility of a host.

Mutants of Erwinia aroideae deficient for specific amino acids have been used to study the correlation between virulence and the presence of the required nutrient in the host tissue [Garber (36); Garber & Schaeffer (37)]. It was concluded that the mutants attacked slices of certain vegetables only when adequate amounts of the required nutrients were present at the infection site and that the mutants were nonpathogenic if the concentration of the

required nutrient was too low to support good growth. These conclusions were apparently based on the untested assumption that conditions favorable for growth were equally favorable for the production of pectolytic enzymes.

From similar experiments, Kline et al. (59) concluded that the nonpathogenicity of certain mutants of Venturia inadequalis deficient for specific substances was caused by their inability to obtain them from the resistant hosts. Mutants deficient for choline, riboflavin, pyrimidines, arginine, histidine, and methionine were used. The criterion for pathogenicity was the ability to incite lesion formation and to sporulate on apple leaves. Histological studies showed that all of the mutants were able to penetrate the cuticle of susceptible and resistant hosts and to become established as microscopic infections, but further development depended mainly upon the addition of the required nutrients from outside the host. Growth of the mutants on leaf sap showed that the required substances, with the possible exception of histidine, were present in the leaves but were not available at the infection site in sufficient amounts for continued development of the parasite. Pathogenicity was restored wholly or partially to six of the eight biochemical mutants by daily addition of the required nutrient to the surface of inoculated apple leaves. The choline and histidine mutants were rendered pathogenic by adding the needed substance to the vascular system of the host plant.

The availability of nutrients at the infection site would depend upon the concentration in the host tissue, the rate of utilization by the parasite, and the rate of translocation from surrounding cells or tissues. Shaw et al. (89) found only slight accumulation of radioactive carbon and phosphorus around lesions of necrotrophic parasites such as Botrytis, Pseudomonas, and Helminthosporium. However, four days after incoulation of wheat and barley leaves with Puccinia graminis and Erysiphe graminis, and sunflower with P. helianthi, there was rapid accumulation of radioactive materials, particularly in the mesophyll cells below the invading mycelium. This was more pronounced in the susceptible than in the resistant type of infection. The authors pointed out that it is essential for successful "obligate" parasitism, that the nutrients be supplied by or transported to the invaded cells at a rate high enough to maintain their normal metabolism in the presence of the parasite.

Permeability and osmotic pressure of host cells.—The successful parasite must be able to absorb nutrients and water from the cells of its host. The nectrotrophic parasite normally performs this task by first killing the host cells and releasing the cell contents. The biotrophic parasites, on the other hand, must overcome the semipermeability of the host cell membrane, which normally holds nutrients within the cell, without greatly injuring the protoplasm. Thatcher (100) found that an increase in permeability of host cells surrounding the invading hyphae of several rusts was constantly associated with susceptibility. The cells of this rust had a higher osmotic value than did the surrounding cells of the susceptible host. Evidence also suggested that a decrease in cell membrane permeability is a factor which tends to confer resistance, probably by retention of solutes and possibly water by the host

cells. Thatcher proposes the theory that the rust fungi secrete at least two enzymes capable of acting differently on the cell membrane of the host cells, changing its permeability. If one of these enzymes becomes inactivated by some substance in the host cell, the permeability would be increased or decreased, depending on which enzyme was affected, and thus the host would become more susceptible or more resistant.

Differences in osmotic pressure appear to be the underlying mechanism of resistance of lettuce to *Erysiphe cichoracearum* and of peach to *Sphaerotheca pannosa* [Schnathorst & Weinhold (84)]. Resistance was directly correlated with the osmotic values of the cell sap. Solutions of sucrose with osmotic values equal to those of the resistant hosts inhibited uptake of water by conidia.

Hypersensitivity.—The term hypersensitivity was used first by Stakman (97) to describe the rapid death of a limited number of wheat cells around the site of infection by Puccinia graminis. This rapid reaction prevented further growth of the fungus and, as a result, varieties of wheat showing hypersensitivity were highly resistant or immune. The term has since been used to describe rapid reactions of several hosts to a number of other patho gens. Several cytological studies of the hypersensitive reaction have been made [Allen (3); Humphrey & Dufrenoy (52); Rice (77); Smith (93); Stakman (97)].

The sequence of events from the time of penetration until death of the host cells and pathogen is not well understood. The theory that the pathogen starves because it cannot obtain required specific nutrients has been proposed [Chester (21); Leach (64, 65); Wellensiek (114)]. However, evidence for the starvation theory is not conclusive. Sharp & Emge (87) succeeded in transplanting infected tissues of resistant wheat leaves containing mycelium of Puccinia graminis tritici to leaves of susceptible plants. The mycelium soon grew out into the healthy susceptible tissue and developed sporulating lesions within seven days.

Humphrey & Dufrenoy (52) attributed the death of host cells of the oat plant to depletion of phosphorous by *Puccinia coronata*. Establishment of invading hyphae was believed to depend upon the release of phosphorus by the host cells. If they became severely depleted in this element, phenolic compounds in the cells were changed by dehydrogenation to toxic quinones by an enzyme system of the tyrosinase type. The host cells survived only if the dehydrogenase system converted the toxic quinones back to nontoxic polyphenols. Whenever the quinones were not rehydrogenated, they catalyzed further oxidations, inhibited respiration, and brought about death of the cells in local areas. These authors conclude, "The interrelationship between host and parasite as evidenced by resistance or susceptibility seems largely dependent on the rate of dehydrogenation of polyphenols to quinones."

A rapid hypersensitive reaction occurs in varieties of red clover resistant to Erysiphe polygoni [Smith (93)]. Penetration of haustoria into resistant cells

caused a severe antagonistic reaction. A rapid disorganization of the cytoplasm began at the point of penetration and spread to the entire cell. Both the infected cell and the invading hypha were soon dead. In some cases a few surrounding host cells were also killed, resulting in highly resistant plants. A slower intermediate reaction occurred in moderately resistant plants, and there was no killing of cells in the susceptible varieties.

The reaction of barley leaves to penetration by *Erysiphe graminis* was not as severe as in the case of the clover mildew. The successful establishment of the parasite appeared to be directly related to the number of haustoria penetrating the epidermis of the host within a given infection site [White & Baker (115)]. Resistance appeared to result mainly from the necrogenic reaction of mesophyll cells to the presence of haustoria in the epidermal layer. The rate of the necrogenic reaction was considered to be the deciding factor determining resistance. The authors explained the collapse of the mesophyll cells as a result of toxin produced by the invading pathogen and the degree of resistance upon the sensitivity of the mesophyll cells to the toxin. Susceptible plants were said to be tolerant to the toxin.

Hypersensitive reactions of the necrotic fleck type have been described for hosts of *Phytophthora infestans* [Gallegly & Neiderhauser (33); Greenham & Müller (47); Müller (72)]. Resistance of this type appears to be attributable primarily to the speed and severity of the reaction of host cells to the invading parasite. However, Ferris (29) concluded that there was little difference in rate of the hypersensitive reaction in susceptible and resistant derivatives of *Solanum demissum* attacked by *P. infestans*. It is generally believed that certain metabolic products of the invading parasite quickly alter the metabolism of the surrounding host cells of resistant plants, resulting in the occumulation of toxic phenolic compounds which are fungistatic or fungicidal, Müller (72) pointed out that the hypersensitive reaction of potato to infection by *P. infestans* is not specific and that the potential to react in this way is probably present in many angiosperms.

Using a technique of inoculating potato tuber slices of different thickness with zoospores of *Phytophthora infestans*, it was demonstrated that the activity of about 10 cells surrounding an infection site was essential for a high degree of resistance [Tomiyama et al. (103)]. The polyphenol content of thick slices was greater than that of thin slices 12 to 48 hr. after inoculation. Other work has demonstrated in leaves of resistant potato varieties a marked increase of polyphenols and in activity of polyphenol oxidase, suggesting that the oxidase system functions as a protective mechanism against *P. infestans* [Rubin et al. (78)]. Increased resistance to *P. infestans* of potato and tomato plants treated with streptomycin was attributed to an indirect effect on host metabolism, since the antibiotic was known not to affect the parasite directly. Higher concentrations of streptomycin were usually correlated with greater polyphenolase activity. It was concluded that streptomycin provides protection by means of its effect on the polyphenol-polyphenolase system of the host plant [Vörös et al. (109)].

A similar explanation of resistance of *Impatiens bulsamea* to certain fungi has been offered by Sproston (96). Spores of *Alternaria tenuis*, *Botrytis allii*, and *Monilinia fructicola* penetrated the epidermal cells; but soon a dark stain appeared and development of the pathogens ceased, suggesting the presence of polyphenols which were converted into toxic quinones, several of which were isolated. Differences in rate of necrotic reaction of varieties of bean to infection by *Colletotrichum lindemuthianum* were believed to be correlated with resistance [Leach (65); Tochinai & Sawada (102)]. The cells of susceptible varieties were not killed until the mycelium had spread widely through the host tissue.

Resistance of barley to Helminthosporium gramineum also appears to result from a form of hypersensitivity [Skoropad & Arny (92)]. Stripe symptoms developed in susceptible varieties only after the pathogen had made rapid invasion into leaf tissue and the chloroplasts had disintegrated. In resistant varieties the host cells collapsed in advances of the hyphae after two or three layers of cells had been penetrated. Both host cells and parasite died in local areas. Kuc et al. (60, 61) found that slices of potato, carrot, and turnip produced substances inhibitory to Helminthosporium carbonum and other fungi following inoculation with these fungi. Two inhibitory substances were separated chromatographically and identified as chlorogenic and caffeic acids. Corn plants showed different resistance reactions to three species of Helminthosporium causing leaf blight [Jennings & Ullstrup (54)]. Resistance to H. turcicum apparently was located in the xylem and acted to inhibit growth, H. carbonum penetrated both resistant and susceptible plants at the same rate, but infection in resistant varieties was confined to the epidermal cells, with little or no growth into the mesophyll. Reaction to H. maydis was intermediate. A hypersensitive reaction of certain tissues of crucifers and lettuce infected by Pellicularia filamentosa (Rhizoctonia solani) was also reported [Flentje (30)]. High light intensity favored the reaction.

Interesting results were obtained with Helminthosporium victoriae and Puccinia coronata on oats [Litzenberger (68)]. Varieties that gave a hypersensitive resistant reaction to P. coronata were highly susceptible to H. victoriae and to low concentrations of the toxin of the latter. There is some evidence that the hypersensitive reaction to P. coronata was caused by a toxin comparable to, or possibly the same as, that produced by H. victoriae. Since the host cells surrounding the rust hyphae were killed, the spread of the hyphae was prevented and the host was resistant. Death of host cells favored further development of H. victoriae, and thus the host was highly susceptible to this pathogen. The production of two toxins by H. victoiae, one highly specific and highly active, the other nonspecific with low activity was reported by Luke & Wheeler (69). Krupka (62) has called the specific toxin victorin. Pringle & Braun (74) also reported two toxic compounds produced by H. victoriae. One, believed to be previously undescribed, was called victorinine and the other was probably a peptide.

Phenolic compounds, either already present in uninfected host tissue or

produced as a result of invasion by a parasite, are believed to be important in determining resistance of potato tubers to *Streptomyces scabies* [Johnson & Schaal (55); Schaal & Johnson (81)], apples and pears to *Venturia inaequalis* and *V. pirina*, respectively [Kirkham (56); Siebs (90)], and apples to *Sclerotinia fructigena* [Byrde (19)]. Several polyphenols were found to accumulate in sound tissue of sweet potato near injured tissue attacked by *Ceratocystis fimbriata*. These included chlorogenic and caffeic acids [Uritani & Miyano (106)]. From other work it was concluded that resistance to *C. fimbriata* was caused in part by the action of ipomearone (an abnormal metabolite) by interfering with phosphate metabolism [Uritani & Akazawa (105)].

Unidentified inhibitory substances have also been shown to be important in resistance. Turner (104) found that two varieties of Ophiobolus graminis differing in pathogenicity to oats also differed in ability to grow on expressed oat sap. The type variety, which did not cause lasting infection of oats, was inhibited by the sap, whereas the variety avenae, pathogenic to oats, was able to grow in the sap. Inhibition was not caused by a lack of nutrients. The inhibition of growth of mycorrhizal fungi by pieces of living tissue of orchids has been reported [Burges (16); Gäumann & Jaag (43)]. Hawker et al. (51) described a similar situation in which a strain of Pythium ultimum penetrated roots of Allium and in its parasitic stage became established in a manner similar to an endotrophic mycorrhizal fungus. Later the hyphae were destroyed, presumably by antibiotic substances formed by the host. Resistance of pea varieties to species of Mycosphaerella was correlated with the presence in the host of substances which inhibited pycnidial formation [Sörgel (95)].

Factors affecting pectolytic enzymes.—The pectolytic enzymes produced by soft-rot pathogens cause dissolution of the middle lamellae and at the same time cause death of host cells surrounding the invading hyphae. This was called the enzyme theory of parasitism by Brown (15). Characteristic of this type of parasitism is the diffusion of enzymes into the host tissue and bringing about the well-known "action in advance." Resistance factors may act either to prevent the production of specific enzymes or to inhibit their activity. Brown (15) has recently reviewed the conditions affecting production of pectolytic enzymes by several pathogens. It will be necessary to present only a few of these conditions here.

The detailed properties of pectolytic enzymes produced by different organisms may not be the same [Brown (14); Echandi & Walker (24)]. Sclerotinia sclerotiorum produced both pectin methyl esterase and polygalacturonase when grown on wheat bran, while Erwinia aroideae produced pectin depolymerase and little pectin methyl esterase. The enzymes of S. sclerotiorum. S. fructigena, and Botrytis cinera have their greatest activity in acid substrata, while the pectic enzymes of Pythium debaryanum, Phytophthora erythroseptica, and various soft-rot bacteria have optima in the alkaline range. The enzyme of E. aroideae requires the calcium ion for activity, while that of

B. cinerea does not [Brown (15)]. Pectolytic enzymes of S. fructigena are believed to be inhibited by phenolic compounds in injured apple tissue (Kirkham (56)].

The substrate may not be favorable for the production of specific enzymes. Both Botrytis cinerea and Pythium debaryanum grew well on potato decoctions, but only the medium of the former pathogen gave active pectolytic enzyme preparations [Brown (15)]. When P. debaryanum was inoculated into wounded potato tuber, a rapid rot resulted. However, Gupta (49) found that both potato decoction and sap from frozen tubers were suitable for production of protopectinase. Enzyme production was not related to mycelial growth. It was further demonstrated that enzyme preparations from Botrytis cinerea failed to attack normal subturgid potato tissue. When the tissue was soaked in water, rendering it completely turgid, it was readily decomposed by a small amount of enzyme [Fernando & Stevenson (28); Mishra (71)]. On the other hand, an enzyme preparation from Erwinia carolovora rotted both turgid and subturgid potato tissue but was less active in the latter. The rate of diffusion of the enzyme appears to be important.

Botrytis allii, which does not normally attack apple, produced a rot readily when a small quantity of various nitrogen sources was added [Brown (14)]. Apparently, resistance of apple to this fungus is attributable to its low nitrogen content. Presumably the pathogens rotting apples produced pectolytic enzymes at lower concentrations of nitrogen than are required by B. allii.

FACTORS MODIFYING HOST RESISTANCE

This section deals with some factors which, for the most part, only modify the growth or pathogenic activities of the parasite after it has become established in the host. Among them are host metabolism, host nutrition, and environment. These factors are closely interrelated and their combined effects may be greater than that of a single factor.

Host metabolism.—One of the most common effects of parasitism is a change in respiration rate of the host tissue. It is reported that the respiration rate of plants infected with obligate (biotrophic) parasites is almost always increased, but the same is not always true for the necrotrophic parasites. A correlation between glycolic acid oxidase activity and the degree of resistance of wheat leaves to Puccinia graminis tritici may mean that this enzyme plays an important role in host-parasite relations [Kiraly & Farkas (58)]. A similar view was expressed previously by Sempio (86), who considered the action of photosynthesis—glycolysis—respiration as important in determining the degree of resistance of wheat leaves to infection by Erysiphe graminis. This is the basis of his theory of "metabolic resistance." Sempio believes that there is a well-defined type of metabolic resistance linked with the ratio of anabolism and catabolism of the host plant and that these processes are markedly influenced by the environment.

On the other hand, the only substances found to break down resistance of

tomato plants to Fusarium wilt were those that inhibited the activity of respiratory enzymes [Walker & Stahmann (113)]. Since respiration of a susceptible tomato plant declined as soon as it became diseased, it was concluded that the fungus functions as a pathogen by bringing about interference with the respiratory enzyme systems of the susceptible host. It has been suggested that resistance to Fusarium depends upon continuous metabolic activity of the tomato plant and that substances many accumulate and become toxic to the pathogen [Gothoskar et al. (45); Scheffer & Walker (83)]. This type of resistance is a feature of the entire plant [Scheffer (82)].

Using rusted and mildewed cereal leaves, Shaw & Hawkins (88) demonstrated an increase in indolacetic acid content in susceptible tissue and a decrease in infected resistant tissue. The level of the auxin was controlled enzymatically by indolacetic acid oxidase. Since indolacetic acid is known to increase cell membrane permeability, these authors suggest that this fact may account for Thatcher's (100) finding increased permeability in susceptible infected host tissue and decreased permeability in resistant plants. Indolacetic acid may play a role in determining whether host nutrients move continuously toward infection loci or whether translocation is prevented.

Host nutrition.—The great amount of work done on the effects of host nutrition on disease development and resistance makes it impossible to review all of the relevant literature in the available space. One general conclusion applicable to the majority of host-parasite relations studied is that different nutrients available to the host plant have variable effects on resistance to different pathogens. Host nutrition may affect the structure of the plant or the metabolic products of the host, or it may affect the pathogen directly, being either favorable or unfavorable to disease development. For the most part the greatest effect is indirectly on the parasite through the nutrients or other metabolites in the host cells. Heavy applications of fertilizer to crop plants in the field, or additions of excess nitrogen to green plants under controlled greenhouse conditions have generally resulted in increased severity of disease, while low nitrogen has generally increased resistance [Kirkham (56); Last (63); Simons (91); Stakman & Harrar (98); Van Gundy & Walker (107); Walker et al. (111)].

A balanced host nutrition appears to be of great importance in determining the degree of resistance to numerous pathogens. Soybean plants grown in sand culture with deficiencies of calcium, magnesium, iron, sulfur, nitrogen or phosphorus were more susceptible to severe infection by *Rhizoctonia solani* than were plants grown in the presence of all these elements [Castano & Kernkamp (20)]. Walker and his associates (34, 35, 111, 112) have presented a series of papers on the relation of host nutrition to disease development. Increase in salt concentration generally retarded development of cabbage yellows. Low nitrogen and low phosphorus resulted in less disease, while low potassium tended to increase disease development. The rate of development of *Verticillium* wilt of tomato was increased, as a rule, with increased nutrient

concentrations of the host solution. This followed the trend determined previously for bacterial canker of tomato and ring rot of potato but was opposite to the trend determined for *Fusarium* wilt and bacterial wilt of tomato.

It is assumed that an increase in nitrogen supply available to the host plant results in an increase in both total nitrogen and in the soluble amino acids that would be more easily available to a parasite. Actually, only a few investigators have followed up their observations and have made nitrogen analyses of higher plants to determine whether this conclusion is correct [Gallegly & Niederhauser (33); Gassner & Franke (38); Samborski & Shaw (79); Van Gundy & Walker (107)]. There is also some evidence that concentration of carbohydrates and the carbon-nitrogen ratio in the host tissue are important in determining the rate of growth of the pathogen [Bird (8); Hurshovetz (53); Manners & Gandy (70)]. Periods of higher resistance in oats to Helminthosporium avenae and potato to Phytophthora infestans were found to correlate with those of most rapid growth and low percentage of total carbohydrate [Grainger (46)]. Regarding a specific compound, Sempio (85) observed that of the several monosaccharides and disaccharides tested which maintained healthy green color of bean leaves attacked by Uromyces appendiculatus, only sorbose prevented growth of the fungus [reported by Allen (2)].

In seeking answers to some of the puzzling problems of parasitism and resistance, a relatively new approach has been used recently. By selecting certain mycoparasites it is possible to study various types of parasitism and different host-parasite relationships without the complicating process of photosynthesis in the host plant. It is also possible to provide the host fungus with a variety of media and even to change the host medium as desired during growth. Experiments to determine the effects of many factors can be carried out under rigidly controlled conditions in a minimum of time and space. Some of the pioneer work was done by Ayers (4), who studied parasitism of Dispira cornuta on species of Mucorales. An agar medium high in nitrogen favored susceptibility of certain hosts, while on media high in glucose and low in nitrogen the same hosts were resistant. Only special media high in nitrogen permitted growth of this parasite in the absence of a living host.

Calcarisporium parasiticum was recently described as a new biotrophic parasite on some species of *Physalospora* and related fungi [Barnett (5)]. Resistance of host species varied greatly and was not correlated with growth of the host. A highly susceptible species, *Physalospora obtusa*, was heavily parasitized on a wide variety of media, while resistance of *P. ilicis* was increased as the concentration of glucose was increased, and decreased in greater amounts of yeast extract or glutamic acid [Barnett & Lilly (6)]. The ratio of carbon to nitrogen also affected resistance.

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Host media high in nitrogen also favored parasitism of species of Mucorales by *Piptocephalis virginiana* [Berry (7)], and *Penicillium notatum* by *P. xenophila*, and also resulted in higher concentrations of soluble nitroge-

nous compounds in the host mycelium (unpublished data). In other studies, parasitism of *Mortierella pusilla* by *P. virginiana* was prevented or greatly delayed by daily additions of glucose to the culture medium.

The specific nitrogen source, such as different ammonium compounds and different amino acids, available to the host had a marked effect on the degree of resistance or susceptibility, although paper chromatography failed to reveal in the host mycelium any specific amino acid that could be considered as the determining factor [Berry (7)]. Of 10 host species tested, only Mycotypha microspora and Thamnidium elegans were highly susceptible on ammonium sulfate. Only Rhizopus nigricans was resistant on casein hydrolysate and glutamic acid. The pH of the medium had little effect on growth of the parasite.

Parasitism of certain species of Phycomycetes by Rhizoctonia solani was affected by temperature, light, and composition of medium [Butler (18)]. Good vegetative growth of both host and parasite appeared to be a prerequisite for infection. Isolates of the parasite varied greatly in ability to attack Rhizopus sp. and Pythium debaryanum.

Acidity of host cells.—There seems to be little evidence that acidity of the host cell sap in itself is of much general importance as a resistance factor acting directly against a parasitic fungus, although in some highly acid fruits parasites may be inhibited. However, cell sap acidity is important in its effect on the activity of various enzyme systems [Brown (14, 15)]. Reddy (76) reported a close correlation between corn cob acidity and resistance to infection by Nigrospora. Smith et al. (94) pointed out the possible significance of the hydrogen ion concentration in the conversion in the host of phenolic compounds to the more toxic quinones following invasion by pathogenic fungi. Increases in pH of healthy tissue surrounding invaded tissue, which would favor extended growth of the pathogen, have been reported [Boyle (11); Venning & Crandall (108)].

Environmental factors.—Environmental factors may modify resistance either by direct effect on the pathogen or indirectly through host metabolism. It is often difficult to evaluate the importance of environmental factors or to determine how they act. Probably some of them alter the host metabolites that are available to the pathogen. Griffith et al. (48) point out that knowledge of the host-parasite-environment relationship should be the first step in determining the basis for resistance. They found that at temperatures averaging 20° C. all wheat plants of a susceptible variety were susceptible to bunt and all plants of a resistant variety were resistant (on the basis of smut ball formation). However, at 10° C. resistance broke down in 58 per cent of the inoculated heads of the resistant variety. Expression of resistance in this case was a result of inhibition of teliospore formation, rather than growth of the parasite. Further studies indicated that the quality of light is a factor in the expression of certain genes for resistance of wheat to bunt [Zscheile (118)].

Postinoculation environment had a greater effect on development of

bacterial wilt of tomato than did preinoculation environment [Gallegly & Walker (34)]. Environment also altered the host-parasite reaction to differences in nutrition. In one environment, bacterial wilt of tomato was reduced in high nitrogen and increased in low potassium, while in another environment it was reduced by low potassium [Gallegly & Walker (35)].

SUMMARY

Resistance of plants to infection by microorganisms is based upon the genetic composition of the host, and expression of the resistance genes may be influenced by numerous factors. The principal factors are aggressiveness of the parasite, presence or absence and availability of specific nutrients at the infection site, presence or absence of specific inhibitory substances, metabolism of the host including the activity of numerous enzyme systems, host nutrition and especially the carbon-nitrogen ratio, and environment.

Resistance to penetration may be either mechanical or chemical, while resistance to invasion or production of disease is mainly chemical or nutritional. The nature of resistance reactions of the host is largely determined by the initial stimulus provided by the parasite and its secretions and the ability

of the host to respond.

The theory of specific nutrients required by races of biotrophic parasites is attractive and would explain many host-parasite relations, but concrete evidence in support of this theory is scarce. Another theory which seems to explain active resistance in many plants is based upon the belief that there is present either before penetration or, more commonly following penetration, certain toxic phenolic compounds, which appear to be commonly associated with the hypersensitive reactions. The typical reaction initiated by the invading parasite appears to involve the transformation of nontoxic phenolic compounds into toxic quinones which are fungistatic or fungicidal to the invading hyphae and which may also be responsible for the death of the host cells. It is not clear at present whether these toxic phenolic compounds are the cause or the result of the death of the host tissue and the invading hyphae. A biotrophic parasite, if not killed by the toxic compounds, is then surrounded by dead host tissue in which it cannot survive. A necrotrophic parasite may also be inhibited or killed by the toxic substances. In either case the host is highly resistant. If, however, the continued development of a nectrotrophic parasite is favored by the dead host tissue, the host may be highly susceptible.

An unbalanced host nutrition low in nitrogen tends to make many hosts more resistant. This is probably caused by insufficient nitrogen available for either growth or enzyme production by the parasite. Factors favorable for growth of a parasite are not necessarily favorable for the production of disease. Resistance factors may act either against growth of the parasite or against its disease-producing power.

Mycoparasites as test organisms should be useful and well adapted to nutritional and environmental studies of basic principles of parasitism and resistance, without the complicating process of photosynthesis in the host. In future investigations it appears that the main efforts should be directed toward determining the chemical and metabolic bases for resistance and their relation to genetics of both host and parasite. This will require the use of modern techniques and equipment in the hands of research workers well trained in biochemistry. Such investigations, if well planned, of long duration, and specifically directed toward these ends, should add greatly to the knowledge and understanding of the nature of plant disease resistance.

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BACTERIA AS PLANT PATHOGENS^{1,2}

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For the first half century following the discovery that bacteria could cause disease in plants, the phytopathogenic bacteria were studied mainly in relation to disease, and primarily by pathologists. Their methods were often inappropriate adaptations of mycological procedures, and their outlook was, quite properly so, clinically oriented. Hence, many of the contributions to the earlier phytobacterial literature consisted of a characterization of a disease, accompanied by a picture of the pathogen which was incomplete—to say the least.

A few farsighted pioneers succeeded in wedding phytopathology with bacteriology to the benefit of both disciplines. Most notable by any standard was Smith, subject of a recent factual biography (146), whose four books (161, 162) and hundreds of articles and monographs which are summarized in a biographical memoir (96), are the actual foundations of this hybrid subject. Many of Smith's associates achieved deserved distinction; of these, Elliott must be singled out for the meticulous attention lavished on two editions (54) of her Manual of Bacterial Plant Pathogens. The decades of devoted service by Stapp in welding together the two faces of bacterial phytopathology, will always be commemorated in his writings; the monumental Bakterielle Krankheiten (165) and the superb introductory Pflanzenpathogene Bakterien (166) are by far the best comprehensive modern treatises in these fields. The efforts of Dowson are summarized in his useful Plant Diseases Due to Bacteria which has recently appeared in a second edition (47). From the German translation (93) of Israilski's Bakterielle Pflanzenkrankheiten, it will be seen that the fruitful admixture of phytopathology with bacteriology accounts for a large portion of the substantial development of bacterial phytopathology which has occurred in eastern Europe. The writings of Riker, Link, Braun, Ark, and their associates over the past quarter century, many of which are mentioned in context, similarly provide an impressive documentation of the beneficial effects accruing to an amalgamation of bacteriology and phytopathology. Purposely saved by the reviewer for the last-but-not-least position in his highly abridged, personal, and subjective roster of those pioneers whose stature permitted them to stride boldly across the chasm between bacteriology and phytopathology, is his predecessor at this reviewing task (24). The scholarly product of Burkholder's dual am-

¹ The survey of the literature pertaining to this review was concluded in January, 1959.

² The following abbreviations will be used: DNA (deoxyribonucleic acid); DPN (diphosphopyridine nucleotide); DPNH (diphosphopyridine nucleotide, reduced form); TPNH (triphosphopyridine nucleotide, reduced form); PD (pectin depolymerase); PE (pectinesterase); PG (polygalacturonase); PP (protopectinase).

bassadorship is exemplified by the treatises on phytopathogenic bacters, prepared for the seventh and preceding editions of Bergey's Manual of Determinative Bacteriology.

The postwar years have been ones of unprecedented activity in the area of phytopathogenic bacteriology. The viewpoint has shifted, precisely as it had in medical microbiology at the turn of the century, from the clinically oriented aspects to basic studies on the pathogens. In conformity with the trend in other biological sciences, an emphasis on biochemistry and metabolism will be noted. Substantial advances have taken place in taxonomy, genetics, toxins, bacteriophagy, serology, pathogenesis, antibiotic control, and other areas. Limitations of space or the reviewer's competence preclude critical evaluation of several active fields. Furthermore, while the parasitic bacteria which cause root tumors of leguminous plants are traditionally "offlimits" to phytopathology, the reviewer pleads only lack of the space (but not the temerity!) to consider the voluminous and controversial literature on "Rhizobium as a plant pathogen." The hundreds of reports of phytopathogenic bacteria on "new" hosts or from previously unreported localities, simply cannot be managed here; Stapp's treatise (165) is sufficiently comprehensive to aid in a literature search, and the indices of Elliott (54), Review of Applied Mycology, Zeitschrift für Pflanzenkrankheiten (Pflanzenpathologie) und Pflanzenschutz, Bibliography of Agriculture, and Plant Disease Reporter are similarly useful.

From that which remains, the reviewer has salvaged two intertwined main topics: I. Systematics or "What Kinds of Bacteria are the Phytopathogens?"; and II., Phytopathogenicity or "What are Some of the Bases of their Pathogenicity?" A good deal of the peripheral and central clutter is thereby swept under the rug—along with unrecognized nuggets—to await the next reviewer's excavation.

SYSTEMATICS

THE GENERA

As related in Elliott's review (53), the early phytopathologists usually named bacterial phytopathogens in accordance with the systems of Migula, Lehmann and Neumann, or Smith; no hesitation was evidenced in placing pathogens in genera with related nonpathogens. Later, probably stemming from the lack of contact between the bacterial systematists and the phytopathologists, a type of insularity developed in the taxonomy of these bacteria. This culminated in the inexplicable and unexplained concept, expounded by a committee of the Society of American Bacteriologists (205), of taxa wherein the phytopathogenic bacteria were to be segregated from other bacteria solely on the basis of phytopathogenicity. Thus, the first (1923) edition of Bergey's Manual of Determinative Bacteriology contained in the family Bacteriaceae, a tribe Erwinieae, described as "plant pathogens" which was, in turn, divided into two genera: Erwinia ("Motile rods, possessing per-

itrichous flagella. The rods are white and a few species form pigment.") and *Phytomonas* ("Rods, yellow and white, motile and non-motile, the motile species possessing either mono- or lophotrichous flagella. May or may not form yellow pigment.").

Decidedly strange taxonomic bedfellows were thrown together by this Erwinieae concept, which gained the remarkable and negative accolade of the following terse acknowledgement (96) from the usually verbose gentleman— Erwin F. Smith—who was to be commemorated: "... the species I have mentioned ought not to be put into one genus (Erwinia) simply because they are plant parasites." By 1930, Burkholder (21), who had set up a comparative study on a grand scale, had sorted out the major groups of bacteria which had previously been lumped into Phytomonas; further refinements are seen in his 1939 paper (23), which suggested division of the genus Phytomonas into several "groups" whose affinities to genera of nonpathogens is specified. With the recognition that Phytomonas was homonymous with a group of protozoan flagellates in apparent conflict with a resolution of the Second International Congress of Microbiology (52), new generic names for Burkholder's groups were desirable. These were forthcoming from Dowson (44, 45, 46), Conn (32), and others, leading to the reassignment of the bacterial phytopathogens in the sixth (1948) and seventh (1957) editions of Bergey's Manual of Determinative Bacteriology to the following genera: Agrobacterium (gall-inducing and saprophytic Rhizobiaceae); Bacillus (sporeforming rods); "Bacterium" (Gram-negative rods of doubtful systematic position); Corynebacterium (Gram-positive and Gram-variable rods with coryneform tendencies, including a few motile species); Erwinia (pectinolytic and nonpectinolytic phytopathogenic Enterobacteriaceae); Pseudomonas (nonpigmented and green pseudomonads); Streptomyces (mycelial); Xanthomonas (slime-forming, yellow pseudomonads). This arrangement, which is used with little or no modification by many workers, including Elliott (54), Stapp (166), Israilski (93), Weiss & Wood (200), White (202), and Dowson (47), is by no means universally accepted, as will be seen from the examples which follow.

Waldee (199) has suggested that the pectinolytic soft-rot Erwinia species be placed in a new genus Pectobacterium, thus effecting separation from the biochemically and pathologically different group centering around Erwinia amylovora. This suggestion, although not officially adopted in the seventh edition of Bergey's Manual, has had wide acceptance. In adopting this Pectobacterium-Erwinia split, Dowson (47) injudiciously included the gall-forming Agrobacterium species in the redefined genus Erwinia. Hellmers (79) has recently brought forth the suggestion, in connection with his outstanding comparative study of several Pectobacterium species from ornamental plants, that groups of related bacteria be set off in subgeneric categories which he calls stirpes (sing. stirps). "Within such a stirps a type species is then designated on the usual priority principles, and the rest of the deviating isolate types of the stirps may then be characterized as varieties of the type

species, even if determination of these varieties is based only on one or a few—but reproducible—biochemical deviations from the other 'members' of the stirps." Based upon his investigation and this principle, he referred the several pectobacteria which he studied to only two species—a move which the reviewer applauds.

Some dissatisfaction has been expressed with the placement of the motile phytopathogenic coryneform bacteria in the genus *Corynebacterium*. The origin of this practice has been traced by Conn & Dimmick (33), and Clark & Carr (28) have suggested the possibility that some of these organisms are re-

lated to soil Arthrobacter species.

The leading Rumanian phytobacteriologist, Săvulescu (157, 158), followed the generic treatment of Bergey's Manual but added a genus Burkholderiella based entirely upon the formation of blue pigment particles by the single species, usually called Corynebacterium insidiosum. It has since been shown by the reviewer (176) that this blue pigment, indigoidine, occurs in bacteria differing considerably from C. insidiosum and that production of the pigment is subject to marked fluctuation—leading one to question the

wisdom of establishing a genus on this single criterion.

Magrou & Prévot (122, 123) proposed placement of the Gram-negative phytopathogens in several genera of a family Pseudomonadaceae, which they emended in a manner which takes it beyond the bounds of a coherent taxon. The nonpigmented phytopathogenic pseudomonads were removed from Pseudomonas to a new genus Phytobacterium, reserving the former for green-pigmented pathogenic and nonpathogenic pseudomonads. These French workers resurrected, without its original meaning, the designation Aplanobacter for Gram-negative, nonmotile, nonsporulating phytopathogens. The foregoing, plus Xanthomonas, Agrobacterium, and Erwinia are all placed in their version of the family Pseudomonadaceae, together with some equally inappropriate nonpathogenic neighbors. The Gram-positive, nonsporulating phytopathogens are distributed in the genera Bacterium (if motile), Corynebacterium (if nonmotile and coryneform) and Eubacterium (if neither motile nor coryneform). Essentially this same system was adopted by Hauduroy (78) in the second edition of Dictionnaire des Bactéries Pathogènes.

Krasil'nikov's determinative handbook (112) places the phytopathogenic bacteria with nonphytopathogenic relatives in the genera Mycobacterium (two coryneforms), Pseudobacterium (most of the coryneforms), Pseudomonas (white, green and yellow pseudomonads), Bacterium (nonpigmented Erwinia), Chromobacterium (pigmented Erwinia), Actinomyces (mycelial),

and Bacillus (sporeformers).

In Yugoslavia, Tešić disposed of the genus problem by adopting the non-committal label *Bacterium* for all 180 of the phytopathogenic species discussed in his 1949 monograph (191) and 1953 review (192). However, in his 1955 critique (193) of Krasil'nikov's system, Tešić heaps high praise on the Russian's classification, although he does suggest use of *Corynebacterium*

(rather than Mycobacterium) and Aplanobacter (rather than Pseudobacterium).

Patel & Kulkarni (134) have returned to a variation of the Erwinieae idea by proposing the separate family Phytobacteriaceae to include all the phytopathogens. While the Indian phytopathologists have accepted Xanthomonas, Agrobacterium, Pectobacterium, and Erwinia, they have created a genus Chlorobacter for the phytopathogenic green-pigmented pseudomonads, adopted Phytobacterium for the phytopathogenic nonpigmented pseudomonads, and returned to Aplanobacter for the nonmotile coryneforms.

THE SPECIES PROBLEM

Whereas a certain stability and rationality, albeit not worldwide, has been achieved in the generic arrangements, absolute chaos still reigns on the specific level. This is an inevitable concomitant of the general practice, when reporting a previously undescribed disease, of presenting an inadequate description of the "new" bacterial species with usually no, or only feeble, attempts at direct bacteriological comparison with possibly related species. What is even more deplorable-and much less understandable-are the equally feeble attempts to explore rigorously the host range of the "new" species. In fact, it seems to the reviewer that the usual emphasis lay in "proving" the uniqueness of the "new" species merely by reporting its isolation from a unique host. It is, therefore, not particularly surprising to a contemporary microbiologist, when these hundreds of "species" are scrutinized comparatively, to discover a chaos of synonomy that no bacterial taxonomist-regardless of his splitter tendencies-could endure. The variability in bacteriological traits exhibited in a direct side-by-side comparison of individual clones ("isolates") of a particular "species" is frequently as great as that shown among different "species." This has been documented from the earliest days to the present, although many of the writers overlook this obvious interpretation of their own data by their slavish and uncritical acceptance of the "new" host-"new" species cliché. The old song, "This bacterium differs from all the phytopathogens listed in Bergey's Manual; hence, it is a new species," has indeed acted as a powerful hypnotic!

Host specificity.—The firmly entrenched "new" host-"new" species doctrine has resulted in the publication of dozens of new "specific" names each year. The problem is considerably aggravated by an attitude that the "new" bacteria, even when observed to be similar in bacteriological characteristics to named species, somehow deserve designation as distinct species merely because they had been isolated from different host plants. Where true differences in phytopathogenic capabilities actually exist, it would be permissible under the International Code (92) to use subspecific epithets. The reviewer, cognizant of the subjective and imperfect nature of bacterial speciation (127, 128), and bowing to custom and practical usage, might even tolerate the promotion of truly unique phytopathogenic varieties and special forms to the rank of a sort of "practical" species. Unfortunately, in entirely

too many cases the effort made to determine the host range of "new" bacterial species was as nonexistent or ineffectual as was bacteriological comparison. Recalling the ancient adage concerning the peculiar vulnerability of inhabitants of vitreous structures, the reviewer hastens to declare his own culpability (177, 179). As will be seen from the following examples, the common errors and omissions include absence of direct comparison, failure to control the choice of horticultural varieties used in testing pathogenicity, unconcern over the preparation and condition of the inoculum, indifference to the site and mode of inoculation, lack of attention to the physiological and environmental situation of the host during the incubation period, and bias in the reading and evaluation of the pathological response.

Despite their bacteriological similarity, Xanthomonas geranii was named as a new species distinct from X. pelargonii because the former did not appear to be pathogenic to pelargonium plants. By using nonresistant horticultural varieties, controlled conditions of inoculation and incubation, and rigorous evaluation of the total symptomatology, the two bacterial "species" have now been shown (182) to infect one another's hosts and thus is confirmed the bacteriological and serological evidence that they are identical.

The definitive study of plant actinomycoses by Hoffmann (87) reveals that the alleged multiplicity of phytopathogenic *Streptomyces* species stems from inadequate direct comparison of the individual isolates of a pathologically and physiologically elastic microorganism. Hoffmann concludes that both potato scab and beet scab are caused by *S. scabies*, and that most of the other so-called species implicated in these diseases are either variants of that

organism or nonpathogenic contaminants.

The cause of soft rot of carrots was described in 1901 and named Bacillus carotovorus, and a year later the cause of potato blackleg was described and named Bacillus atrosepticus; both would nowadays be referred to the genus Erwinia (or Pectobacterium). In the intervening half century there have been dozens of articles alleging or denying the identity of the two bacterial species mainly on the basis that only the strains isolated from potato blackleg could cause blackleg symptoms; assorted minuscule cultural differences were also invoked. This hiatus was finally resolved a few years ago by the observations of Hellmers & Dowson (80) that typical blackleg symptoms were produced in potato, tomato, and tobacco by strains of both species when they were inoculated directly into the vascular bundles of young plants; inoculations into parenchyma regions did not lead to infection. This brings to mind the experiences of Last (114) and Logan (118) who demonstrated that the reaction of cotton plants to contact with Xanthomonas malvacearum was heavily affected by the site of inoculation, as well as by concentration of inoculum, technique of inoculation, and many other factors which are not usually considered when performing pathogenicity tests. Observations of this sort are so numerous and varied, that the preparation of a summary and critical evaluation of them would be an extremely useful undertaking.

The situation of Pseudomonas syringae provides an outstanding example

of the gross synonomy attendant upon inadequate comparative work. At the last count, some 18 differently named "species" had been shown by careful comparisons to be the same bacteriologically and pathologically [Elliott (54); Clara (27); Wilson (203); Fuchs (63)]. A good deal of the credit for the clarified taxonomy of *P. syringae* can be attributed to the eminently reasonable attitude, expressed by Wilson (204) 25 years ago in connection with a pseudmonad from a new pear disease in California: "Obviously, the organism mentioned herein cannot be assigned a name until it is compared with similar organisms. It is becoming more and more evident that new species created from studies of the disease of a single host are often unjustified."

Entirely too often the preconceived idea that a "new" species was being described led to subjective evaluation of the pathological response in favor of the "new species." So, for example, in attempting to differentiate the filbert pathogen Xanthomonas corylina n.sp. from the bacteriologically identical walnut pathogen X. juglandis, it was deemed necessary—since both pathogens can infect the leaves of both host plants—to fall back on hair-splitting distinctions involving differences in size of lesions on young walnut fruits or in susceptibility of stems of a particular age (125). Then there is the remark that the cabbage pathogen Xanthomonas campestris really does not infect horseradish (the "specific" host of a "new" pathogen, X. campestris var. armoraciae) because a few leaf spots developed only under conditions very favorable for the bacteria (119). Why, indeed, should conditions in a

pathogenesis trial not be very favorable for the bacteria!

The foregoing examples could be multiplied many times. There is really no need to labor the point further in view of the recent direct evidence that the potential host range of xanthomonads is very broad. Dve's splendid doctoral dissertation (48) and preliminary note (49) relate that, after four successive passages through bean plants, progressive disease of bean could be caused by cultures of Xanthomonas alfalfae, X. begoniae, X. beticola, X. betlicola, X. campestris, X. campestris var. armoraciae, X. citri, X. geranii, X. hyacinthi, X. juglandis, X. malvacearum, X. nigromaculans f.sp. zinniae, X. papavericola, X. pelargonii, X. poinsettiaecola, X. pruni, X. punicae, X. ricinicola, X. translucens, and X. vesicatoria. Some of these organisms retained virulence for their original host plants after acquiring bean virulence. These results provide experimental support for the idea that the relatively few true species of phytopathogenic bacteria exist in many "special forms" characterized by a quite elastic adaptability to particular plants. The ease with which the latent pathogenic capability is expressed, can, as discussed in the final section below, account for the origin of "new species" on "new hosts." It explains many things, except contentions (201) to the effect that the xanthomonads are "remarkably specific in their host reactions" and that "there is no possibility of error in using the host as a species determiner."

Determination of species.—A decade ago, the reviewer and his erstwhile professor (25) stated that it was essentially impossible to determine phytopathogenic bacteria to species by the usual procedures of the bacteriological

laboratory; this opinion is now reiterated. Since, to use Rahn's phrase (137), it is a "practical impossibility" to carry out plant inoculation tests which would provide positive identification on a pathological basis of a microorganism from an unknown habitat, a stalemate exists in locating a rationale for determination of these microorganisms. As a working hypothesis, it was assumed (25) "that where there is a true difference in pathogenic ability, some other type of difference should be demonstrable—be it cultural, biochemical, metabolic, serological, or some other category." It was not, of course, inferred that these hypothesized differences are necessarily related causally to the differences in pathogenicity. In any case, search for such differences has been undertaken in a number of institutions; while nothing revolutionary has taken place during the past decade, some glimmer of light will be seen in several of the sections which follow.

SEROLOGY

It is now clear that proper application of serological methods has considerable value in the identification of phytopathogenic bacteria on some subgeneric level. This viewpoint has been championed by Stapp and by Israilski, whose extensive personal observations are summarized in their respective books (93, 165, 166). On the other side, there have been comments on the subject ranging from cautionary to caustic, largely from investigators who do not employ serological methods (22, 47, 201). Three excellent papers by Elrod & Braun (59) relate that five compact serological groups can be demonstrated in the genus Xanthomonas, provided the typical haptenic mucoid exudate, which confused the serological picture in earlier work, was eliminated by cultural or chemical treatment. Rosenthal & Cox (148, 149) have made an interesting start in analyzing the serological makeup of the phytopathogenic corynebacteria, as has Friedman (62) in his study of the phytopathogenic pseudomonads. Nine isolates of Erwinia amylovora, obtained from different localities and hosts, had identical agglutination and agglutinin absorption reactions (55); on the other hand, considerable heterogeneity is seen in the antigenic makeup of Agrobacterium spp. (11) and of Bacterium stewartii (18, 121). Elrod's report (56) on the serological heterogeneity of the soft-rot Erwinia spp. has been substantiated by the painstaking serological analysis carried out by Okabe & Goto (132) which reveals that 180 cultures of Erwinia carotovora could be divided into 12 serological groups based on flagellar antigens. In the light of these findings, one must question Nováková's report (130), based upon slide agglutination tests of cultures isolated from diseased potatoes, that all the isolates which could rot potato slices (and only those cultures) were agglutinated by the Erwinia antiserum. It is, of course, possible that the strains tested in this series were serologically homogeneous because they originated from a limited geographical area.

It should be evident from the above sampling that serology of bacterial phytopathogens has not yet attained a state of perfection which enables

unequivocal identification by the use of this technique alone. Nor is it yet possible to correlate serological properties with pathogenic capabilities. The methodology has, however, proven to be an important adjunct in solving various practical determinative and diagnostic problems; properly employed and interpreted, it will inevitably assume increasing significance particularly as further basic knowledge on the serological properties of these bacteria accumulates.

BACTERIOPHAGY

It has been suggested that bacteriophage lysis be used for identifying phytopathogenic bacteria as well as for diagnosing and controlling bacterial diseases of plants. Since the entire subject of bacteriophagy in phytopathology has been painstakingly reviewed in 1956 by Stolp (184), the present treatment will be briefer than is warranted by the potential importance of the theme. Phages with activity against phytopathogens, including representatives of every genus, have been isolated by workers in all parts of the world. Because the specificity of the bacteriophage is particularly germane to its utility in taxonomic determination, one should note that the known phytobacterial phages range from strictly strain-specific to almost genusspecific. For example, some of the Xanthomonas translucens phages isolated by Katznelson & Sutton (100) and the Pseudomonas solanacearum phages described by Okabe & Goto (131) were strain-specific. On the other hand, Xanthomonas pruni phages have been reported which were remarkably "species"-specific: only the 13 X. pruni cultures out of 212 strains of Xanthomonas spp. tested by Eisenstark & Bernstein (51) were lysed by their X. pruni phage. The two phages isolated by Fulton (65) from wildfire- and blackfire-diseased tobacco lysed most strains of the causal agents of the two diseases, Pseudomonas tabaci and P. angulata. A few strains of the two bacterial "species" were resistant to one or the other, but not to both, phages; four "species" of phytopathogenic pseudomonads (but not several others) originating from plants other than tobacco were susceptible to these phages. At the other extreme, Sutton, Katznelson & Quadling (188) recently described a polyvirulent phage that lyses numerous "species" of Xantho-

Using the principle that an increase in the number of phage particles in a given suspension can be considered presumptive evidence of the presence of that phage's host bacterium in that suspension, Katznelson, Sutton and their collaborators (99, 101, 187, 188) have been able to detect various phytopathogenic bacteria in infected plant material without the necessity of isolating the bacteria. This technique, used with proper safeguards, shows considerable promise as a routine diagnostic tool in plant pathology.

Most of the recent work suggests that phytobacterial phages are similar in properties to the phages of nonpathogenic bacteria. One of Fulton's two *Pseudomonas* phages consisted of characteristic tailed particles, while the second preparation contained tailless round or irregular particles. The

Erwinia carotovora phage of Chapman, Hillier & Johnson (26) similarly reveals a mixture of the familiar tailed type with tailless and multi-tailed particles, some with irregularly shaped bodies. The polyvirulent Xantho-

monas phage had the typical tailed morphology.

As with serology, bacteriophagy would seem to be a potentially important and practicable determinative aid in phytopathogenic bacteriology. Here, again, we have only a bit of raw material rather than an assembly line of the finished product. The fact that a single phage preparation lyses bacterial strains originating from more than one kind of host plant ("different bacterial species") supports the contention that such lysed bacteria are, in fact, biologically closely related—rather than the defeatist attitude that bacteriophagy per se is inapplicable to determination of the bacterial phytopathogens.

METABOLISM

Despite the obvious advantages which would accrue from a thorough understanding of the metabolism of phytopathogenic bacteria, surprisingly few comprehensive contributions have been made in this area during the period under review. What is available leads one to the general conclusion that the fundamental metabolic processes of these pathogens are identical with those of nonpathogenic relatives. For example, a preliminary abstract by Zajic, De Ley & Starr (213) reports that intermediates of the tricarboxylic acid cycle were metabolized by intact cells and cell-free extracts of Corynebacterium insidiosum. On the basis of the enzymatic activity of cellfree extracts, it is apparent that C. insidiosum utilizes sugars via the hexosemonophosphate shunt and the pentose cycle. Katznelson (97, 98) has presented the results of a preliminary survey for the presence of certain key enzymes involved in glucose metabolism. He concludes that species of Xanthomonas, Pseudomonas, and Agrobacterium metabolize glucose by the shunt pathway and either by the 6-phosphogluconate system, the pentose cycle or both; Erwinia species may use either the glycolytic or the oxidative route; phytopathogenic Corynebacterium species appear to use the pentose cycle. A more detailed investigation of glucose-6-phosphate oxidation by Xanthomonas phaseoli led Hochster & Katznelson (83) to conclude that the hexosephosphate is oxidized chiefly by Zwischenferment, followed by an Entner-Doudoroff split to triosephosphate and pyruvate. Some of the hexosephosphate is degraded by the pentose cycle; the tricarboxylic acid cycle, but not the classical glycolytic route, appears to be operative.

Kraght & Starr (110) have reported that the end products from the fermentation of glucose and galacturonic acid by *Erwinia carotovora* were identical to those in a typical *Aerobacter* fermentation: lactate, succinate, formate, acetate, ethanol, 2,3-butylene glycol, and carbon dioxide; hydrogen was not formed by the particular strain used, and ethanol was produced only from glucose and not from galacturonate. More recently, Starr, De Ley & Kilgore (178) and Kilgore & Starr (103) traced the separate enzymatic steps in the

catabolism of uronic acids by *E. carotovora*; galacturonate or glucuronate (but not mannuronate) is isomerized by uronic isomerase to the keturonic analogue which is, in turn, reduced by keturonic reductase (with DPNH or TPNH) to the corresponding p-hexonate, followed by dehydration and phosphorylation to 2-keto-3-deoxy-6-phosphogluconate, which is finally split to triosephosphate and pyruvate. This hitherto unknown metabolic pathway is not unique to phytopathogens; an identical scheme was unravelled independently by Ashwell, Wahba & Hickman (10) in *Escherichia coli*. This route is not followed by *Pseudomonas syringae* which oxidizes galacturonate to mucate by means of the recently described uronic dehydrogenase and DPN [Kilgore & Starr (104)].

Sutton & Starr (186) have reported that Erwinia amylovora grows aerobically in media containing mineral salts, glucose, and nicotinic acid, but cannot grow anaerobically in such media unless L-malate or fumarate is added; anaerobic growth is improved by the further addition of a mixture of amino acids. Suspensions of cells, grown either aerobically or anaerobically, are capable of anaerobic dissimilation of glucose; the major end products are carbon dioxide, ethanol, and lactate, with traces of formate, acetate, and acetoin. The relative amounts of the major products varied depending upon the pH, the kind of buffer used, and the manner in which the cells were grown. Fermentation of 1-, 2-, or 6-C¹⁴-glucose yielded unlabelled carbon dioxide; these data, which are consistent with the operation of the Embden-Meyerhof pathway of glycolysis, were substantiated by demonstration of the enzymes of this pathway in cell-free extracts of E. amylovora.

Reference to other investigations related to this area will be found in the sections on genetics, phytotoxic substances, and nutrition. Evaluation of the many isolated remarks on metabolism of phytopathogenic bacteria in contributions whose main theme lay elsewhere will have to await a special treatment of this subject.

GENETICS AND VARIABILITY

Colonial variation.—The usual "wild-type" strain of bacterial phytopathogen, as isolated from an infected plant, occurs in the mucoid or smooth phase with such regularity that the existence of rough forms of phytopathogenic bacteria was denied by some earlier workers. The thorough work of Link & Hull (117) and Sharp (159), thirty years ago, provided distinct demonstration of colonial variation and its relationship to virulence and serology for members of almost every genus of the bacterial phytopathogens. Ark's (6) classical study of variability in Erwinia amylovora included observations on the relatively low virulence of rough types. His papers on the mutagenic action of naphthalene and of uranium compounds (7, 8) contain striking illustrations of colony variations in Xanthomonas juglandis and Corynebacterium michiganense. Corey & Starr (34) described a series of colonial variants in Xanthomonas phaseoli, showed that there was a parallel increase in quantity of polysaccharide slime produced and in virulence for

bean in progressing through the series $R \rightarrow S \rightarrow SM \rightarrow M$. Furthermore, they (35) were able to demonstrate deoxyribonucleic acid-mediated interconversions of the colony types, which led to the postulation that polysaccharide production (and thereby colony type and virulence) was controlled by three genes each with a nonfunctional allele. The fully mucoid strain was depicted as bearing all three functional genes, the rough cells as containing only the three nonfunctional alleles, and the two intermediate types as carrying both functional and nonfunctional genes.

Pigmentation.—Many of the phytopathogenic bacteria are chromogenic and this trait is used as a determinative character in most taxonomic schemes. Scattered observations on alteration in pigmentation have been made from the earliest times. Bryan (20) isolated spontaneously occurring white and pink strains of the normally yellow Corynebacterium michiganense, and similar color variants were obtained by Ark (7, 8) from cultures treated with acenaphthene or uranium salts. Saperstein, Starr & Filfus (156) showed that the major pigments of C. michiganense are carotenoids and that the parent and the several color mutant strains possess distinctly different carotenoids. An association of the carotenoids with protein particles has been demonstrated (155), and the occurrence of the rare ketonic carotenoid canthaxanthin was reported in one of the color mutants (154). Starr & Saperstein (181) elucidated the qualitative and quantitative changes which occur in the carotenoid pigments and colony color of Corynebacterium poinsettiae when the concentration of the nutrilite, thiamine, in the culture medium is altered. A similar situation was worked out in C. michiganense (12, 156). The reviewer (176) recently reported variation in the production of the extracellular blue pigment indigoidine by selected strains of Corynebacterium insidiosum; variability in the color of the cells was also noted.

Transformation of streptomycin resistance.—The normally streptomycin-sensitive cells of Xanthomonas phaseoli can be transformed to streptomycin resistance by deoxyribonucleic acid from streptomycin-resistant cells [Corey & Starr (36)]. A phenomic delay was encountered after transformation of streptomycin resistance during which the cells are genotypically streptomycin-resistant and phenotypically streptomycin-sensitive. The cells used as the source of DNA were one-step mutants resistant to at least 2000 μ g. streptomycin per ml., and the transformants for streptomycin resistance were also resistant to at least 2000 μ g. streptomycin per ml. The addition of antiserum or a preparation of unrelated DNA prior to addition of the specific DNA prevented transformation, thus suggesting that strain competence may involve a DNA receptor site at the cell surface. Evidence was presented to the effect that all of the cells are capable of being transformed and that cellular competence represents the fraction transformed for the character under investigation.

Unpublished observations by the same investigators show that the streptomycin-resistant DNA of X. phaseoli can be introduced into a wide variety of Xanthomonas "species"; again reinforcing the reviewer's contention that

the number of true species in this genus is limited. Further comments on genetics of the bacterial phytopathogens will be found in the final section on alteration of virulence.

CYTOLOGY

Following the publications of Stoughton (185) almost thirty years ago on what he interpreted as nuclear structures and zygote formation in Xanthomonas malvacearum, there were no reports on the cytology of the bacterial phytopathogens until Robinow mentioned (145) his otherwise unpublished observations with de Garcia Cabral concerning the chromatinic bodies of X. begoniae and X. malvacearum.

Not until the last few years has there been a thorough investigation in this field; namely, the highly interesting work on the cytology of star-formation in the genus Agrobacterium. The original observation of Stapp & Bortels (164, 167) showed that the formation of stars in A. tumefaciens results from the assembling of several motile rod-shaped cells. After protoplasmic coalescence, the star disintegrates, the individual cells begin to divide, and the young cells swarm. Braun & Elrod (16) published electron micrographs of these stars and attempted to explain the origin and nature of these aggregates. In a few instances, they observed what appeared to be a fusion of the Feulgen-positive material at the center of the aggregates, leading to the cautious suggestion that this represents a simple form of sexuality. With the aid of nuclear stains and electron microscopy, Stapp & Knösel (168) investigated the developmental cycle of the star-forming bacteria A. tumefaciens, A. radiobacter, Rhizobium pisi, R. trifolii, and a marine bacterium for which they proposed the name Agrobacterium steilulatum n. sp. In all cases, they observed a union of the cells at the center of the star, followed by a fusion of the individual "nuclei" to a single central "nucleus." After division of this central nuclear substance, the cells pass through a period of nuclear increase and finally divide into short swarm cells. In a second publication, Stapp & Knösel (169) report that observations of living cells with the phase microscope confirm in every detail the results obtained earlier with dead cells, and in a later article (170), they describe an almost continuous observation of the star cycle. Here they used various histo-enzymatic methods, all of which serve to confirm their earlier interpretation that star formation is a true process of copulation; in a separate note (171), they deny categorically the claims, such as Heumann's (81), that there is actual zygote formation. Knösel (108), after a thorough investigation of the nuclear condition in A. stellulatum, concluded that there is no mitotic nuclear condition but, rather, the formation of chromatinic granules, the form, size, and arrangement of which he describes and discusses. These investigations were continued by Knösel & Günther (109) who confirm the nonexistence in Agrobacterium of mitosis in the fashion of higher organisms. However, a process analogous to mitosis seems to take place wherein there occurs a differentiation of the nuclear substance into three granules which, after increas-

ing in volume, divide to form six granules; three of these bodies come to lie next to each other and fuse to form the daughter nuclei.

Douglas, Robinson & Corke (43) have reported osmotically sensitive, protoplastlike structures produced by the action of lysozyme on the mycelium of *Streptomyces scabies*. These "protoplasts" did not possess the phage receptor sites present on the original hyphae, but did contain cell wall antigens.

PHYTOPATHOGENICITY

The reactions of the host plant to infection by bacteria can be somewhat arbitrarily categorized as wilt, necrosis, abnormal (usually hypertrophic) growth, and rot; however, the symptom complex is rarely limited to a reaction of a single category. One might, with similar didacticism, subdivide into several elements the capacity of a pathogen to cause disease, Gäumann's (74) division into affinity, aggressiveness, and pathogenicity provides a useful concept, which causes one to focus attention separately on (a) compatibility with the host (affinity); (b) the capacity to invade the host plantthat is, to enter and inhabit it, overcome its resistance, and multiply in it (aggressiveness); (c) the ability of the organism to evoke disease (pathogenicity). From the stimulating reviews of Brian (19) and Kern (102), it will be seen that practically no experimental work with phytopathogenic bacteria bears directly upon this distinction. Nonpathogenic bacteria, presumably aggressive in the Gäumann sense, have been reported by Tervet & Hollis (190), Hollis (88, 89), Sanford (153), and others; these investigators describe the occurrence of bacteria in apparently healthy (that is, symptomless) plant tissues. Relevant to this theme is the report by Allington & Chamberlain (3) that Pseudomonas glycinea and Xanthomonas phaseoli, when introduced into leaf tissues of a host normally considered to be immune, multiplied but did not induce symptoms under their experimental conditions. Alternatively, the very common saprophytic pectinolytic bacteria, which can rot excised plant tissues but not spread through the intact plant, might be viewed in Gäumann's terms, as potentially pathogenic organisms which lack the aggressiveness of a "complete" pathogen. What little is known about aggressiveness of phytopathogenic bacteria can be gleaned from Kern's (102) review. On the other hand, considerable effort has been expended in recent years in relating phytopathogenicity to discrete bacterial substances ("toxins") which induce disease symptoms in the host plant. The term "toxin," in the phytopathological literature, is used in the general sense of a poisonous substance generated by the pathogen regardless of its chemical nature.

PHYTOTOXIC SUBSTANCES

Wilt-toxins.—Although earlier writers believed that wilt symptoms followed plugging of the xylem vessels by cells of the pathogen, current opinion has shifted toward the idea that microbial products, termed wilt-toxins by Gäumann (75), rather than the cells themselves, interfere with water trans-

port and bring about wilt. Certain polysaccharides produced by phytopathogenic bacteria in vitro possess wilt-inducing properties; generally these substances are much less host-specific than the microbes which form them. McIntire, Peterson & Riker (120) have isolated, from a virulent strain of Agrobacterium tumefaciens, a water-soluble glucosan, molecular weight about 3600, which can cause wilting of tomato cuttings. The wilt-inducing polysaccharide has been found by Hodgson, Riker & Peterson (84, 85, 86) in attenuated strains of A. tumefaciens; in fact, any of a number of plant and microbial polysaccharides, synthetic polyethylene glycols, and polyvinyl alcohols similarly induced wilting of tomato cuttings, leading to the conclusion that they are toxic because they interfered mechanically with the transpiration system. An entirely different polysaccharide, molecular weight 19,000,000, which was isolated by Leach et al. (115) from the typical slimy exudates formed by Xanthomonas phaseoli grown on glucose, caused wilting of tomato, sunflower, and bean, despite the fact that only bean is infected by this xanthomonad under natural conditions.

Feder & Ark (61) isolated Boivin-type endotoxins from A. tumefaciens, X. phaseoli, and Erwinia carotovora. Wilting of tomato and sunflower cuttings was brought about by these endotoxins and by polysaccharides derived from them. It might be noted, parenthetically, that similar endotoxic preparations from a large assortment of phytopathogenic bacteria, including species of Xanthomonas, Erwinia, Pseudomonas, Corynebacterium, and Agrobacterium, were shown by Zahl, Starr & Hutner (212) to have considerable tunity for implanted mouse sarcoma. This digression provides an opportunity to mention the report of Elrod & Braun (58) that the tobacco pathogen Pseudomonas polycolor can cause fatal infections in small laboratory animals; conversely, the animal pathogen Pseudomonas aeruginosa can induce leaf necroses, soft-rots, and wilts in various plants.

Although their observations were not specifically directed towards wilting, the results of Corey & Starr (35) with colonial transformants of X. phaseoli are relevant in that a parallel increase in production of alcohol-precipitatable polysaccharide slime and in virulence for bean occurred as

the colony type was altered from $R \rightarrow S \rightarrow SM \rightarrow M$.

Husain & Kelman (90) and Winstead & Walker (206) have related the wilt-inducing ability of *Pseudomonas solanacearum* to the possession by that brown-rot bacterium of pectinolytic and cellulolytic enzymes. These enzymes attack the pectic and cellulosic substances of the plant vascular tissue, liberating gums which block the water transport of the plant. Harris (77) has put forth an essentially similar idea to explain the wilts caused by *Bacterium stewartii* and *Erwinia tracheiphila*. Pectinolytic enzymes are commonly formed by bacterial phytopathogens and, as Ammann (5) has shown, water-soluble methylcellulose is attacked by constitutive enzymes present in filtrates of *Erwinia aroideae*; a preliminary survey by Goto & Okabe (76) reports that growing cultures of a considerable number of phytopathogenic bacteria can liquefy nutrient carboxymethylcellulose gels.

Necrotizing toxins.—In the wildfire disease of tobacco, caused by Pseudomonas tabaci, the most characteristic symptom is formation of angular, brown, necrotic spots on the leaves; these spots are surrounded by a broad chlorotic halo. This halo is free from bacteria, and cell-free filtrates of P. tabaci cultures will produce similar chlorotic haloes if pricked into tobacco leaves. Woolley, Braun and their collaborators (209, 210, 211) have shown that the toxin is a derivative of a hitherto unknown diamino-dicarboxylic acid, β -hydroxy- α , ϵ -diaminopimelic acid, to which they apply the trivial name tabtoxinine. The toxin, which is probably the lactone of α -lactylamino- β -hydroxy- ϵ -aminopimelic acid (211), is very potent; as little as 0.05 μ g. produces necrosis and the chlorotic halo when introduced into a tobacco leaf. Using the unicellular alga Chlorella as a test organism, Braun (13, 15) showed that L-methionine suppresses the toxic effect of the toxin, leading to the supposition that the toxin interferes with methionine metabolism of the plant -an idea which is supported by the observation that a synthetic antagonist of methionine, methionine sulfoxime, induces the same symptoms in tobacco leaves as does Pseudomonas toxin.

Many plants other than tobacco are affected by P. tabaci toxin; alternatively, strains of P. tabaci which have been kept in laboratory culture for long periods, lose the ability to form this toxin, but still infect tobacco plants with the milder symptoms of blackfire disease-allegedly caused by a different species, Pseudomonas angulata. Acquisition of ability to cause the wildfire disease—and, hence, inferentially, to form the P. tabaci toxin—has been described by Clayton (29) and by Johnson (94, 95) in experiments with saprophytic soil strains of Pseudomonas fluorescens which had been applied to water-soaked tobacco plants. Although these conclusions have been criticized by one group of phytopathologists (195), these highly interesting observations are supported by the serological and physiological studies of Reid et al. (138), which reveal the essential identity of P. fluorescens, P. angulata, and P. tabaci. Substances with properties suggestive of P. tabaci toxin have been implicated in bacterial diseases of plants other than tobacco; for example, in wildfire of soybean by Allington (2), in bacterial halo blight of coffee by Costa et al. (37), in halo blight of beans by Waitz & Schwartz (198), and in stone-fruit necroses by Erikson & Montgomery (60).

Hypertrophy-inducing factors.—One group of phytopathogenic bacteria characteristically brings about the formation of tumors, or galls. The extensive literature on crown gall—easily the most voluminous in any area of bacterial phytopathology—can be traced by consulting the publications of Braun (14), Hildebrandt (82), Klein & Link (107), Rack (136), de Ropp (147), Riker et al. (140), Riker & Hildebrandt (141, 142, 143), Riker, Spoerl & Gutsche (144), Rybak (151), and Stapp (165, 166). Intimate details are available on numerous properties of the gall-forming bacteria; many aspects of the host-parasite interrelationship and host response have been meticulously investigated. Nevertheless, the role of Agrobacterium tumefaciens, in transforming normal host cells into tumor cells, is by no means completely

understood. The current consensus has veered away from the concept that A. tumefaciens produces a discrete etiological factor, in favor of assorted hypotheses which invoke multiple causative elements. Braun & Laskaris (17) suggested that there are two phases to gall formation: conversion of host plant cells into tumor cells; and stimulation of tumor cells by indolacetic acid and other auxins to continuous multiplication leading to the formation of galls. All attempts to find the converting principle for the first step have failed. Riker's (139) appraisal of the situation led him to the view, now generally held, that initiation of such pathological plant growth results from an imbalance of a number of important physiological factors, of which products and activities of A. tumefaciens are a part. The magnificent review of Klein & Link (107) presents an analysis of this multiplicity of factors and a hypothesis which accounts quite convincingly for the known facts. The bacterial tumor-inducing principle may be a specific deoxyribonucleic acid which converts the conditioned plant cells (which form by the action of wound substances upon normal plant cells) into incipient tumor cells. These latter, not yet capable of tumorous growth, are acted upon by auxins which are formed by virulent A. tumefaciens (or by the adjacent normal or the incipient tumor cells themselves) to form enlarged promoted cells. These, in turn, are converted into primary tumor cells by postulated agents derived from cells and tissues of the host plant.

Rotting enzymes.—Soft-rot bacteria damage tissues by virtue of an ability to decompose the pectic substances of the plant cell wall. Tribe (194), Fushtey (66), Meyer von Gregory & Wartenberg (124), and Lapwood (113) have discussed the moot point of whether the tissue maceration which follows such decomposition reflects all, or only a part, of the toxigenic capabilities of these bacteria. Despite the excellent general summaries which have been published on the pectic enzymes and pectic substances by Altermatt (4), Demain & Phaff (40), Deuel & Stutz (42), among others, considerable confusion still exists in the terminology for the substrates and enzymes. The classification of Deuel & Stutz (42) will be followed here: pectinesterase (PE) hydrolyzes the methyl ester groups of pectin, ultimately yielding the demethoxylated pectic acid; polygalacturonase (PG), of which several types are known, hydrolyzes the α-1,4'-glycosidic linkages of pectin and pectic acid; in addition, the term "protopectinase" ("PP") will be used for the perhaps hypothetical (40) enzyme which is thought to hydrolyze the poorly characterized insoluble parent pectic substance, protopectin, and form soluble pectin or further breakdown products; and "pectin depolymerase" ("PD") is taken to mean that sort of PG action which results in accumulation mainly of oligouronides, so that relatively little increase in reducing groups accompanies the observed decrease in viscosity of the pectin solutions.

By far the largest part of the literature on the breakdown of pectic substances by phytopathogenic bacteria consists in observations on the lique-faction of nutrient pectate gels by growing cultures, such as reported by Starr (174), Burkholder & Starr (25), Sabet & Dowson (152), and Rudd

Jones (150). Crude enzymes and cultures which macerate slices of plant tissue have come in for their share of attention [Elrod (57); Wood (208); Paton (135); Shishelova (160)]. The fermentation of pectin in culture media has been the subject of investigations by several groups; some have used considerable ingenuity in overcoming the difficult task of preparing a purified pectin and of sterilizing it in a manner which would not seriously alter its composition, others unwittingly used impure pectin or hydrolyzed the pectin in an attempt to sterilize the medium.

Relatively little had been done with purified enzymes from bacterial phytopathogens prior to the past decade; however, the pioneering research of Davison & Willaman (39), Norman (129), and Mills (126) should be men-

tioned.

Erwinia carotovora was reported by Kraght & Starr (111) to have PE based upon the circumstantial evidence of a quantitative liberation of methanol in cultures growing in a high-methoxyl pectin medium. Using the multiple criteria of decrease in viscosity and ethanol precipitability as well as jucrease in reducing value, they were able to show a PG with pH optimum about 5.8 in culture filtrates of pectin-grown (but not glucose-grown) E. carotovora—provided fluoride were present to inhibit the usual rapid degradation of galacturonic acid. Smith (163) showed conclusively that all eight strains studied of soft-rot Erwinia (including cultures labelled as E. carotovora, E. atroseptica, E. aroideae, and E. phytophthora) produced PE, and also PG as determined by the destruction of viscosity and alcoholprecipitability of pectin and pectic acid solutions and by the appearance of chromatographically-detected galacturonic acid and oligouronides. Fujii (64), Akai & Oishi (1), and Ozawa & Okamoto (133) similarly describe substrate-induced PG activity in E. aroideae and E. carotovora and relate that the products of hydrolysis are decomposed vigorously.

On the other hand, using E. aroideae grown in a pectin-free medium. Wood (207, 208) reported that no PE was formed; he did describe "PD" activity which lowered the viscosity of pectin solutions without increasing the reducing value. Similarly, Echandi, Van Gundy & Walker (50) reported "PD" and no PE formed by E. carotovora, E. atroseptica, and E. aroideae. Pectinesterase is usually substrate-induced and its formation is quite sensitive to variation in other cultural conditions (163); hence, failure to find this enzyme could conceivably be related to the use of a culture medium which does not have the proper composition—for example, Wood used a pectin-free medium in which the substrate-induced enzyme, PE, would not be expected to form. Reports that "PD" is formed by Erwinia, which are based solely upon observed decrease in viscosity without detecting a concomitant accumulation of galacturonic acid, have the likely explanation that the lively further catabolism of galacturonate described in Erwinia by Kraght & Starr (110), Starr, De Ley & Kilgore (178), Kilgore & Starr (103), and Fujii (64) proceeded at such a rate as to preclude detection of the galacturonate. Neither in the report of Wood (208) nor of Echandi, Van Gundy & Walker (50) is there any indication that this quite likely event was considered in the experimental design and, hence, one must bear in mind the possibility that any galacturonate which may have been formed was carried past the reducing-sugar state by other enzymes in these relatively impure enzyme preparations. It is, of course, conceivable that soft-rot *Erwinia* spp. have two alternative modes for the breakdown of a substrate so important in their economy as pectin; one by means of substrate-induced PE and PG (Kraght & Starr; Smith; Ozawa & Okamoto), and the second with a constitutive (Wood) or a substrate-induced (Echandi et al.) "PD."

Keeping in mind that really very little is known about the action of soft-rot bacteria on the insoluble "protopectin" as it occurs in plant cell walls, it is still obvious that the pathogenicity of these organisms is related in some way to the production of pectic enzymes. From the survey made by Smith (163), one gains the impression that PE production (which is restricted to pathogens, although not formed by all pathogens), correlates better with pathogenicity than does PG formation. The latter enzyme, while present in all softrot bacteria (perhaps at times in the form of "PP" or "PD"), occurs also in many nonpathogens. The actual disorganization of the plant tissue undoubtedly results from the hydrolytic action of PG (or "PP" or "PD"). However, since PG is formed by many bacteria which are not phytopathogenic, and PE seems, in Smith's survey, to be restricted to pathogens, the question must be asked: What are the possible pathogenic consequences of PE activity? One effect might be to increase the susceptibility of the pectic substances to the enzymatic hydrolysis which is the predecessor of plant tissue maceration. This idea is supported by the statement of Deuel & Solms (41) that the hydrolysis of pectin by fungal PG proceeds more rapidly as the degree of esterification of the pectin is lowered, Smith's (163) re-evaluation of Wood's (208) data on the "PD" of E. aroideae similarly reveals that the initial breakdown and final extent of degradation are much greater for sodium pectate than for the high methoxyl pectin. Many other properties of pectic substances depend on the extent of esterification; Deuel & Solms (41) summarize this dependence: "An increase in the degree of esterification of the carboxyl groups causes an increase in water solubility, dissociation of the acidic groups, viscosity, birefringence of flow, swelling, resistance toward electrolyte coagulation, and alkali lability. The gelling tendency, the stability toward polygalacturonases, and the ion selectivity change regularly with an increasing degree of esterification." Probably many of these factors could be related, directly or indirectly, to the observed correlation of a capacity to rot or wilt plants with an ability to de-esterify pectic substances.

NUTRITION AND PHYTOPATHOGENICITY

The nutritional requirements of phytopathogenic bacteria are of considerable theoretical interest in light of the fact that, during infection, the host plant must provide all the necessary nutrients to a pathogen. With the somewhat naive hope that gross differences in nutritional requirements would

be uncovered, and thus point to a nutritional basis for the host specificity of the individual pathogens, the reviewer and his collaborators (172, 173, 175, 180, 183) surveyed the minimal nutritive requirements of the several genera of phytopathogenic bacteria under conditions of controlled inoculum, carefully cleaned glassware, and pure medium components.

One can conclude from this survey that the gross nutritive requirements for growth of practically any bacterial phytopathogen, can be met by organic substances available in practically any plant. Why, then, are many bacterial phytopathogens limited, at least temporarily, to particular host plants? Lewis (116) has presented an interesting balance hypothesis of parasitism, the gist of which is that "innate immunity is determined by the normal host metabolites that are present and unfavorable to the growth of the parasite, or by the partial or complete absence from the host metabolites of substances that are required by the parasite, or by a combination of these." A similar formulation is offered by Garber (67, 68) as a nutrition-inhibition hypothesis of pathogenicity which invokes nutritional and inhibitory environments in the host and the manner in which they directly affect the fate of an invading parasite. Either of these may or may not be adequate for the growth and virulence of the parasite. "Of the four possible combinations of host environments, only the adequate nutrition-ineffective inhibition environment results in virulence; the other environments result in avirulence." This hypothesis has had fruitful experimental scrutiny [Garber (69); Garber & Hackett (70); Garber & Schaeffer (72); Garber, Shaeffer & Goldman (73)]. Amino acidrequiring auxotrophic mutants of Erwinia aroideae were avirulent for radish and turnip; the return to prototrophy, or addition of the required amino acid to the site of inoculation, restored virulence, or-perhaps more accuratelyrestored aggressiveness. Histidine-requiring strains of E. aroideae were avirulent for turnip, in some cases because of low histidine content of the turnip variety; in other cases, where the histidine content was apparently sufficient for proliferation of the E. aroideae auxotrophs, the presence of inhibitors or antagonists which interfere with the uptake of histidine by the bacteria was postulated. The most recent publication from this group [Garber & Heggestad (71)] relates the avirulence for tobacco of amino acid-requiring mutant strains of Pseudomonas tabaci, and thus adds another example to this interesting model which so profitably depicts nutritional and inhibitory factors in virulence.

Information on the chemical nature of the nutritional and the inhibitory substances is still far from complete. As noted above, we now have a fair understanding concerning the composition of culture media which will support the continued growth *in vitro* of most of the bacterial phytopathogens. Nothing is known about the specific inorganic requirements for growth; the excellent summary of Hutner *et al.* (91) suggests that practicable techniques are now available for scrutinizing the inorganic nutrition of phytopathogenic microbes. Studies of this subject may have important bearing on the unexplained observations that the inorganic diet of the plant has profound effect

upon the susceptibility of the plant to disease. Inhibitory substances have received scant attention except in the burgeoning field of antibiotic control of plant disease [Ark & Alcorn (9); Zaumeyer (214); Tanner & Beesch (189)]. Practically nothing is known about the natural bacteriostatic agents formed by plants. There have been reports alleging that higher plants do, in fact, produce antibiotic substances which inhibit and kill bacteria *in vitro*; however, the reviewer is aware of no systematic examination of the possible role played by such substances in resistance of plants to bacterial infection.

ALTERATION IN VIRULENCE

Since the bulk of evidence available today supports the idea that many of the "species" of phytopathogenic bacteria are bacteriologically similar, an alleged host specificity was invoked to distinguish the "species." Unfortunately for the peace of mind of those who preach this doctrine—and go to remarkable pains to "prove" its validity—the genetic plasticity of the phytopathogenic bacteria in this respect is considerable. Authenticated cases of acquisition and loss of virulence, based upon reproducible and confirmed experimentation, are now commonplace.

A decade ago, Coleman & Reid (30, 31) reported the conversion of virulent Agrobacterium tumefaciens into avirulent A. radiobacter by exposure of cultures of the former to a crude "transforming principle" prepared from the latter. Inoculation of plants with smooth (but not mucoid) strains of A. radiobacter resulted in return to virulence. The mechanism of this genetic alteration has been clarified in two splendid papers by Klein & Klein (105, 106), the first of which shows that a factor capable of transmitting the property of specific host virulence to avirulent strains of Agrobacterium tumefaciens, as well as to A. rubi, A. radiobacter, and Rhizobium leguminosarum, was contained in each of the following preparations: extracts of crown-gall tumor tissue which contain bacteria, a heat-killed suspension prepared from virulent A. tumefaciens, crude nucleic acid produced by this microorganism, and DNA isolated from it. In their second publication, they used a quantitative bioassay for crown-gall tumor formation. The transforming principle was shown to be a DNA, and definitely not a bacteriophage. It was possible to boost the virulence by additional exposure to the deoxyribonucleic acid so that over 30 per cent of the isolates of a stabilized, altered population was significantly more virulent than any of the isolates from the unaltered, recipient population. Klein and Klein suggest that virulence in A. tumefaciens is controlled by multiple loci which regulate the synthesis of a tumor-inducing principle that is effective in plants of a given host range, and that the varying degree of virulence within a particular host range may, in turn, be controlled by multiple alleles at each host range locus.

Van Lanen, Baldwin & Riker (196) have reported that continued cultivation of A. tumefaciens in media containing concentrations of glycine which suppressed growth, invariably resulted in attenuation of virulence. In a second publication (197), they extended the list of virulence-attenuating

agents to include a variety of amino acids, noted the permanence of this attenuation, and recorded the impossibility of distinguishing virulent from attenuated strains by cultural reactions, serological behavior, or enzyme activity.

Dalzell's doctoral dissertation (38) describes the conversion of the smooth phase of the saprophyte Aerobacter cloacae to the mucoid phase of the cucumber pathogen Erwinia tracheiphila by DNA-mediated transformation, as well as by exposure to an extract obtained from healthy cucumber tissue. This latter finding, and particularly Dalzell's interpretation of it as a likely route for alteration of virulence in nature, the reviewer considers very important. For some years, he has been whispering with cautious restraint (mainly in the relatively safe forum of his immediate circle) that a possible in vivo method for acquiring new virulence characteristics is exposure of a related bacterial type to plant tissues. Until recently, this hypothesis could be bolstered only by the following arguments: (a) the acquistion of pathogenicity for tobacco in nonpathogenic pseudomonads—that is, the "creation" of Pseudomonas tabaci—upon introducing these saprophytes into water-soaked tobacco plants; (b) the conversion of avirulent Agrobacterium radiobacter into virulent A. tumefaciens by plant passage; (c) the constant genesis of "new" species of bacterial phytopathogens as evidenced by the interminable stream of reports of phytobacterial disease in "new" hosts; (d) the invariable association, in and around plants, of saprophytic, ectoparasitic, and rhizospheric pseudomonads, coliforms, coryneforms, agrobacteria, sporeformers, actinomycetes, the xanthomonad Bacterium herbicola, and other nonpathogenic counterparts of the phytopathogens; (e) the sudden emergence of phytobacterial diseases where there is no epidemiological history of prior contact with the "specific" causal agent. To these, can now be added: (f) Dye's extensive observations, summarized in an earlier section, on the acquisition of bean virulence—that is, the "creation" of Xanthomonas phaseoli-merely by serial transfer of assorted xanthomonads in young bean plants; and (g) Dalzell's preliminary and unpublished report (admittedly based upon experimental work which requires amplification and extension) that the exposure of a nonpathogenic Aerobacter to cucumber juice "creates" the cucumber pathogen Erwinia tracheiphila.

The presently available information—and perhaps the moral support of equally iconoclastic allies (38, 89)—removes some of the reviewer's hesitancy in reiterating this hypothesis before a wider audience. Unfortunately, what is now known does not permit a decision to be reached on the actual means whereby the bacterium could acquire virulence upon exposure to plant tissues. The various possible mechanisms which are envisioned include, alas!, none which are incontrovertible on the basis of currently accessible facts: (a) stemming from the known receptivity of virulence-affecting bacterial DNA by phytopathogens and related saprophytes, it is conceivable that the plant can donate to a suitable bacterium a compatible plant DNA or other genetic material which alters virulence; (b) perhaps the host plant exerts a

selective action on postulated naturally-occurring virulent individuals in a statistically avirulent population; (c) possibly the plant provides both a mutagenic and a selective action; or (d) some other genetic mechanism or environmental relationship obtains. Certainly, no one can deny that we are facing a major breakthrough in understanding the origin of phytopathogenicity in otherwise ordinary bacteria. Time—and much additional effort—will undoubtedly disclose the actual mechanism; its impact on diagnosis and control of plant disease, quarantine procedures, bacterial taxonomy, genetics, and other aspects of phytopathology and microbiology will inevitably be profound.

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NEMATODES IN PLANT DISEASE¹

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INTRODUCTION

It is the purpose of this review to summarize the current status of our knowledge of the role of nematodes in plant disease, particularly in the period since the publication by Chitwood & Oteifa (1), in 1952. In view of the large volume of literature which has appeared subsequently, many worthy contributions must be omitted and only selected items can be discussed.

Soil nematology, in comparison with related sciences, is still in its infancy. When increased crop yields followed soil fumigation with nematocidal compounds, interest in nematology was stimulated and, during the past few years, this science has received considerably more attention. Taylor (2) has estimated that nematodes cause several hundred million dollars loss each year in the United States alone. As research has continued, we have found that many plant diseases thought to be caused by other factors actually are the result of nematode attack, alone or in combination with other pathogens. The figures on crop damage, therefore, will inevitably increase.

Three main groups of nematodes live in the soil: saprophytes, predators, and plant parasites. The saprophytic nematodes are the most numerous, aiding us by breaking down organic matter and adding the decomposition products to general soil fertility. Predaceous nematodes feed on algae and fungi as well as other nematodes. Plant parasitic nematodes feed on living plants. There is hardly a horticultural crop which does not have one or more nematode species feeding on some part of the plant. Injury caused by nematodes results in various host responses, depending upon the plant and nematode involved. Dropkin (3) classifies these responses as (a) hypertrophy, (b) cell hyperplasia, (c) cell necrosis, (d) dissolution of parts of cell walls, and (e) alterations in the differentiation of cell tissue. Penetration of plant tissue by nematodes may result in secondary infection by other microorganisms, sometimes completely masking the effect caused by the nematodes.

TAXONOMY

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Nematodes are extremely difficult organisms to classify because of their complex anatomy and small size. They have all the organs of the higher animals, except a respiratory-circulatory system. In recent years, the basic concepts of the taxonomists have changed (4). Many nematode groups, previously thought to be a single species, are now considered to be composed of several species or, in some instances, groups of genera.

¹ The survey of the literature pertaining to this review was concluded in October, 1958.

The study of the morphology and systematics of parasitic-type nematodes has proceeded rapidly during the past seven years. All of the new nematodes described have been found associated with plants but, with few exceptions, little is known to their relationship to plant disease. Those which have been established definitely as plant pathogens are discussed in the Pathology Section of this review.

Much work still remains to be done. Some of our most important groups of plant pathogenic nematodes, e.g., the genera *Ditylenchus*, *Belonolaimus*, and *Paratylenchus*, are in a welter of taxonomic confusion.

PATHOLOGY

Absolute evidence of plant parasitism by nematodes is generally lacking. The difficulties of fulfilling Koch's postulates have been well covered by Oostenbrink (5) and by Pitcher (6). A technique for observing nematodes feeding on plants growing under sterile conditions has been proposed by Mountain (7). This procedure has not been widely used because it is difficult and time consuming, and many nematodes will not grow in culture.

The problem, however, is not confined to the fulfillment of Koch's postulates. Nematodes live in a complex environment. As Pitcher (6) has pointed out, it is most difficult to separate the individual components of this environment in order to determine the pathogenic part played by each. Since it is known that the presence of nematodes increases the severity of certain diseases, it is probable that in many cases a combination of nematodes and other microorganisms is necessary to produce primary disease symptoms.

NEMATODES CAUSING PLANT MALFORMATIONS

The genus Meloidogyne (Goeldi).—Of the nematodes causing malformations, the root-knot nematodes belonging to the genus Meloidogyne are the most widely known. The genus is composed of several species which attack at least 1865 different species of plants (8).

In the general life cycle, the first stage larvae hatch from the egg and soon moult into the infective second stage larvae. M. hapla Chitwood larvae are attracted only to growing roots. The apical 2 mm. of root tip repels the larvae, while the next 6 mm. section attracts. This is possibly attributable to the different anatomy and physiology of the two regions (9). On the other hand, Meloidogyne incognita acrita Chitwood has three different major infection courts in sweet potatoes, i.e., young root tips, lateral root ruptures, and the surface of cracks.

After infection the larvae migrate intercellularly and intracellularly to the site of feeding which is primarily in the stele region of cell elongation, cambial zone, and parenchyma (10). *M. arenaria* Neal has been observed in the crowns, petiole, and leaves of African violets, showing that attack by root-knot nematode is not limited to roots (11).

The feeding of nematodes in the genus *Meloidogyne* causes giant cells, abnormal xylem, hyperplastic parenchyma, and cork. *M. incognita* feeds on simple nitrogen compounds broken down by the action of nematode secretions (12).

The typical host response from Meloidogyne infection is gall formation. Infection of roots by Meloidogyne hapla results in a distinctive small gall with lateral roots growing from it (8). The author has observed that the size and shape of the gall with other species depends on the host plant and growing conditions. Dropkin (13) states that there is a relationship between the size of the gall and the number of larvae in the root. He observed small local swellings in tomato roots without larvae inside. The author has observed the same condition in cucumber seedling roots infected with M. incognita acrita. The larvae were more than 0.25 in. away from the swelling in some cases. Myuge (12) grew beans in extracts of galled roots, extracts from healthy roots, and in water. The growth of the bean in the gall extract was initially retarded. However, the toxic effect changed to growth stimulation and, after five days, growth was considerably better than that resulting from the other treatments. Chitwood et al. (14) found that moderate infection with either Meloidogyne incognita or M. javanica Treub significantly increased the growth of peach trees. However, a heavy population of either nematode caused stunting.

After infection the second-stage larvae moult and develop into flask-shaped adults and deposit eggs in a gelatinous sac close to the root surface. Reproduction without males is regular and normal for root-knot nematodes. However, sexual reproduction is known in the genus, mostly occurring when the host plant is under adverse conditions.

The genus Heterodera (Schmidt).—The genus Heterodera comprises a group of nematodes which are generally ectoparasitic. In contrast to many nematode types, the species of this genus are rather specific in their host range. The life cycle is similar to that of Meloidogyne. It differs in that eggs are deposited inside the female body, and upon death, the body of the female becomes a tough resistant cyst which protects them. The genus is one of the oldest plant parasitic groups known, and factors influencing infection and other pathological aspects have been reviewed (1). Tremendous crop damage is caused each year by the two most important species, namely, the golden nematode of potatoes, Heterodera rostochiensis Wollenweber, and the sugar beet nematode, H. schachtii Schmidt.

Recent histological and pathological studies have shown that secondstage larvae of *H. rostochiensis* enter the growing secondary and tertiary roots of the host plant. After infection the cells surrounding the larvae become dense and the cell wall thickens. The third-stage larvae develop in the cortex and stele causing retardation and disruption of vascular elements with some external galling. The formation of giant cells results in dissolution of cell walls (15). Different host species of *Solanum* vary in their reaction. S. dulcamara forms giant cells as well as external swelling. Two other Solanum species show formation of giant cells with out external swelling (16).

Temperature studies with *Heterodera rostochiensis* have shown that maximum development of the parasite occurs at 10 to 18.3°C. (50 to 65°F). (17). The nematode is unable to complete its life cycle at 29.4°C. (85° F). (16).

The sugar beet nematode H, schachtii is established in almost every important sugar beet growing section of the world. Infected beets are small and stunted, having many more roots than normal.

Three other species of the genus which are becoming increasingly important plant pathogens are *Heterodera tabacum* Lownsbery & Lownsbery on tobacco (18), *H. glycines* Ichenoke on soybeans (19, 20), and *H. major* O. Schmidt on cereal crops (21).

The genus Ditylenchus (Filipjev).—The bulb and stem nematode D. dipsaci (Kühn) Filipjev is primarily an endoparasitic nematode which attacks above-ground parts of plants as well as the roots. There are several biological races each of which is limited in the number of plant species attacked (22). For example, a race of D. dipsaci from red clover when transferred to potato caused severely crinkled and malformed leaves. Races from Vicia faba, narcissus, and Plantago lanceolata have had little or no effect on potato (23). Attempts to establish morphological differences among races have not been successful (24).

According to Courtney (25), Ditylenchus dipsaci larvae attack the crowns and leaves of young teasel plants during prolonged moist periods. During the second year, they attack the tissue surrounding the growing point and are carried upward by plant elongation. The teasel head responds to infection by forming a soft-walled mass. As the mass becomes dry the nematodes enter a quiescent stage which lasts for several years under dry conditions. The quiescent nematode becomes active under moist conditions and attacks new plants. Infection of young cereal seedlings proceeds through the stoma into the tissue. After infection, leaves and stems become hypertrophic and deformed (26).

Dunning (27) has observed several interesting effects of the nematode on sugar beets. If the seedling was attacked, leaf distortion and galling occurred. When the growing point was attacked, lateral buds were stimulated to develop. Underground attacks on roots caused cankers. Feder & Feldmesser (28), working with *Ditylenchus dipsaci* infecting narcissus, have found that the "spikkled" areas, and sickle-shaped leaves of infected plants are caused by hypertrophy and hyperplasia adjacent to the areas of nematode attack. They conclude that the "spikkled" areas are homologous with nematode galls.

Galls on the leaves of Calamagrostis were observed to contain all stages of D. graminophila (Goodey) Filipjev. Young galls contained only the nematode, while the other galls contained the fungus Dilophosphora alopecuri in addition to the nematode (29). Spiral-shaped swellings on the roots of

Gramineae were observed to contain up to 60 adult *D. radicicola* (Greeff) Filipjev with thousands of eggs and larvae (30).

Seinhorst (31) was the first to observe that the leaves of potatoes infected with *Ditylenchus destructor* Thorne frequently show deformation. Goodey (23) found that all varieties of potatoes tested, except Epicure, were susceptible to attack by *D. destructor*, and showed some degree of leaf malformation.

The genus Nacobbus (Thorne and Allen).—N. batiformis Thorne and Schuster has been found to attack sugar beets in Wyoming and Nebraska. The damage caused by this nematode is very similar to that caused by the genus Meloidogyne. The life cycle and pathological effects of this nematode infecting sugar beets were worked out by Thorne & Schuster (32). The larvae enter the small roots of sugar beets where they feed and undergo two moults, leaving the root after the second moult. The final moult occurs in the soil, where the larvae become young females and males. The young female enters another root and is established with the head toward the central cylinder. The cells surrounding the nematode enlarge and a gall develops. The young female becomes sac shaped, with the posterior portion toward an opening in the root. The eggs are deposited in a gelatinous matrix outside the body.

The genus Trichodorus (Cobb).—The genus Trichodorus is considered by Christie (33) to be one of the four most important plant parasitic nematodes in Florida. There are two species in Florida, but only one is common and widespread. This species probably attains its greatest importance as a parasite of corn, but beets, celery, cabbage, and tomatoes may also be severely injured. A critical study of the host-parasite relationships of Trichodorus sp. on tomato was made by Rohde & Jenkins (34). They found that the nematode fed primarily on the root tips, puncturing the outermost cells by a rasping motion of the odotostylet. After the cell was punctured, the protoplast shrank from the cell wall. Histological studies indicate that damage to the root is caused by a decreased rate of cell multiplication. No distinct lesions or galls were present, but the lateral roots near the base of the stem were malformed. Some species of the genus apparently have the ability to reproduce on some plants without causing apparent injury. Martin (35) found that an undescribed species of Trichodorus reproduced abundantly on cotton without causing visible damage.

The genus Anguina (Scopoli).—The bent grass nematode, A. agrostis (Steinbuch) Filipjev, has become a serious pest in the states of Washington and Oregon (36). The damage is a reduction of seed production rather than injury to vegetative parts of plants. The larval nematodes remain within the sheaths adjacent to the growing tips through most of the year. In late spring the nematode enters the embryonic flower and matures. Large numbers of eggs are laid, which hatch into larvae inside the seed coat. This activity changes the seed into a dark colored gall. These galls fall to the ground and the larvae can reinfect other bent grass plants. The nematode produces only

one generation per year, and the life cycle is completed within three to four weeks. The nematodes cannot live in moist soil for more than one year in the absence of a host plant.

The wheat nematode, Anguina tritici (Steinbuch) Filipjev, is world-wide in distribution. The principal crops injured are wheat and rye. The larval nematodes attack young wheat or rye seedlings, invading the leaf sheaths and the growing point. Infected seedlings have twisted, curled, or wrinkled leaves the stem may be enlarged at the base, and frequently is bent and stunted. The nematodes invade the developing wheat kernel which then forms a dark gall in place of a normal grain. Within these galls the nematodes reach maturity. After mating the female may lay as many as 2000 eggs. The eggs quickly hatch and the larvae remain inside the gall. When the plant matures, the larvae go into a dormant stage. If the galls are sown with wheat seed or fall to the ground, they soon collapse if the soil is moist. The nematodes, revived by the absorption of water, escape into the soil where they infect young seedlings (37).

Other genera.—Three other nematode genera usually associated with necrotic lesion damage have been shown to produce plant malformation. Radopholus similis (Cobb) Thorne has been shown to cause gall-like formations on the roots of grapefruit seedlings (38). Xiphinema diversicaudatum (Micol.) Thorne will cause root galling of rose, tomato, soybean, okra, cucumber, balsam, peanut, fig, and strawberry (39, 40, 41). The galling is described as an enlargement and curling of the root tip accompanied by necrosis. Van Gundy (42) found Hemicycliophora sp. associated with distinctive galls at the distal portion of the terminal and lateral roots of rough lemon.

NEMATODES CAUSING NECROTIC LESIONS

The genus Pratylenchus (Filipjev).—The study of the endoparasitic meadow nematodes, Pratylenchus spp., during recent years has shown that this genus is one of the most important plant pathogenic nematode groups. In the author's estimation, damage caused by this group exceeds that caused by any other genus. Pratylenchus vulnus Allen and Jensen is the primary pathogen of the root lesion disease of walnuts (43). The same nematode causes a disease of roses characterized by dwarfed, chlorotic plants having stunted, nectrotic root systems (44). P. penetrans (Cobb) causes infected Mazzard cherry trees to wilt; infected roots show a greyish color with many roots dead (45). The same nematode causes severe injury to strawberry, apple, and potato (46, 47, 48).

Abaca roots infected with *P. musicola* (Cobb) Filipjev show lesions ranging in length from 1 mm. to 10 or more cm. The lesions, even in well-advanced stages, are confined to the cortex. The root retains its normal form and the central cylinder remains unaffected. Despite symptoms of attack on the roots, the parts of plants above ground appeared relatively normal (49).

Chapman (50) found as many as 104,500 Pratylenchus sp. per gram of

fresh roots in two-month-old alfalfa seedlings. Root damage was extensive and severe, and the alfalfa stand was severely reduced. Attack by *Pratylenchus* sp. was observed to result in severe root rotting of *Pyrethrum*, *Centurea*, and *Chrysanthemum* (51).

The genus Tylenchorhynchus (Cobb).—The most important species of the ectoparasitic genus Tylenchorhynchus is the tobacco stunt nematode T. claytoni Steiner. This nematode is a devastating parasite of tobacco and causes tremendous damage where it occurs. In addition to tobacco (52), the species has been found to cause damage to azaleas and corn (53, 54). When the corn is in the seedling stage, high populations of the nematode must be present to cause appreciable damage. As the plants become older and develop a more extensive root system, they can apparently tolerate the nematode. Pure cultures of Tylenchorhynchus dubis (Butschli) Filipjev have been shown to cause moderate stunting of cotton and tepory beans (55).

Birchfield & Martin (56) inoculated pure populations of *T. martini* Fielding into sugar cane growing in sterilized soil. In the presence of large populations of the organism, the symptoms of disease were blunt, irregular, and sparse root systems. Though the root systems were severely affected, they did not demonstrate a significant reduction in green weight of the infected over the noninfected plants. The author has observed field experiments in which control of this nematode, by soil fumigation, did not increase sugar cane yields. Evidently sugar cane can withstand severe root injury without

decreased vield.

The genus Radopholus (Cobb) Thorne.—The burrowing nematode, R. similis (Cobb) Thorne, has become increasingly important as the causal agent of spreading decline of citrus. At the present time, a substantial area of citrus is known to be affected with this disease. The nematode attacks the feeder roots causing lesions which later lead to the death of the root. Affected trees are stunted, have smaller and fewer leaves, reduced yields, and wilt abnormally in dry periods (58). The nematode attack is more severe at depths below 20 in., and burrowing nematodes have been recovered from roots growing 12 ft. below the surface of the ground (59). Christie (60) states that beyond any reasonable doubt the disease is caused by the burrowing nematode. He also concludes that the yellows disease of pepper in the Orient, and the spreading decline of citrus are different names for the same disease.

Van der Vecht & Bergman (61) inoculated rice plants growing in sterilized soil with Radopholus oryzae (V. Breda de Haan) Thorne. The nematode caused a decreased in the number of shoots produced in early growth. In some experiments, this temporary reduction of the tillering process was beneficial to the plant. The uninoculated control plants tillered excessively and developed more shoots than could be supplied with necessary nutrients. The infected plants having fewer tillers recovered to yield twice as much rice as the uninoculated control.

The genus Ditylenchus (Filipjev).—The potato rot nematode, D. destruc-

tor Thorne, is widespread in Europe. An endoparasite, it has a limited host range in Canada and the United States. Goodey (23) inoculated clean, healthy potato tubers with *Ditylenchus destructor* and noted that small lesions developed in which the nematodes reproduced. As infection progressed, the areas around the lesions became soft and sunken and the entire tuber eventually rotted. These same effects have been noted on iris and giant snowdrop bulbs (62).

The nematode excretes amylase and a proteolytic enzyme which includes sulfhydryl groups. Respiration increases one and one-half times in potato tissue infected with *D. destructor*, and breakdown of proteins with subsequent coagulation produces necrosis. Also, hydrolysis of starch upsets normal osmosis causing dehydration of infected cells (63).

The observations by Baker et al. (64) indicate that the nematode may not be an obligate parasite of potato, but might feed on the mycelia of associated fungi. They suggest that a species complex exists. There may be one species feeding on potato and another species feeding on fungi. Darling et al. (65) cultured Ditylenchus destructor on undifferentiated tissue from potato, carrot, clover, and tobacco.

The genera Helicotylenchus (Steiner) and Rotylenchus (Filipper).—The spiral nematodes, Helicotylenchus spp. and Rotylenchus spp., derive their name from the position assumed when moulting or dead. These genera probably constitute an important plant parasitic group through they have received little attention.

H. nanus Steiner is able to multiply and live as an ectoparasite on the roots of a number of crop and weed plants. The species has not been found to be pathogenic except on the soybean variety Ogden (66).

Rotylenchus buxophilis Golden² is a primary parasite of boxwood roots. causing numerous nectrotic lesions, some of which may extend well into the cortex (67). R. brachyurus Steiner² is an endoparasite as well as an ectoparasite of African violets and parasitizes the crowns as well as the roots (68). The cells in infected roots are severely damaged and many destroyed. The same nematode reduced the top and root weight of tobacco by 50 per cent (69).

The genus Hoplolaimus (von Daday).—The lace nematodes are widespread in soils throughout the world. They have been observed both as ectoparasites and endoparasites. Since little pathological investigation has been done on this group, their importance as plant pathogens is not well understood. The most extensive work on this group has been carried on in North Carolina by

² It has been called to the reviewer's attention that the spiral nematodes have been divided into several new genera by Andrassy. *Rotylenchus buxophilus* has been changed to *Gottholdsteineria buxophila* (Golden, 1956) Andrassy, 1958, and *R. brachyurus* has been changed to *Scuttelonema brachyurum* (Steiner, 1918) Andrassy, 1958.

Krusberg & Sasser (70). In greenhouse studies, they found that *Hoplolaimus coronatus* Cobb, at populations comparable to those found in the field, caused only slight stunting of cotton plants. They observed that definite root lesions were associated with the feeding of the nematode. Although the nematode fed readily on epidermal and cortical tissues, phloem appeared to be preferred. Phloem parenchyma and phloem elements soon die after infection. Infected phloem tissue appeared to have an alkaline reaction, as evidenced by staining characteristics. Occasionally, xylem elements were infected and produced more tyloses than uninfected elements.

Green peas growing in soil inoculated with *H. uniformis* Thorne developed a yellowing of the leaves. Examination of infected roots revealed discoloration of the vascular tissue, and considerable rotting of the roots (71).

The genus Belonolaimus (Steiner).—The sting nematode, B. gracilis Steiner, is one of the largest phytoparasitic nematodes. It is an ectoparasitic type, feeding on the root tip and along the sides of the host root. It has been reported by various investigators to cause field damage to at least 25 horticultural crops. Christie et al. (72), using relatively pure populations, demonstrated pathogenicity on corn, celery, and strawberry. Necrotic lesions and a stubby root were present on all three hosts. Stunting of all plants occurred and strawberry plants were eventually killed by the nematode attack. Graham & Holdeman (73) did not observe stubby root systems on infected cotton plants. Initial disease symptoms were minute, dark, shrunken lesions along the root axis and tip. In advanced stages roots are severely damaged or destroyed with general stunting of the infected plants.

The genus Dolichodorus (Cobb).—The awl nematode, D. heterocephalus Cobb, is an important plant pathogen in the few areas where it is found. The nematode favors moist conditions and is frequently found in the sub-irrigated areas of Florida. Using pure populations of D. heterocephalus, Perry (74) demonstrated that the nematode caused almost complete root destruction and corresponding stunting of growth of celery, corn, bean, tomato, and pepper plants. In addition, the nematode was capable of feeding on the seed

embryo, thus preventing the seed from germinating.

The genus Criconimoides (Taylor).—Criconimoidies spp., the ring nematodes, are frequently encountered in agricultural soils. Very little information, either biological or pathogenic, is available. Present evidence of their role in plant disease is limited. One undescribed species has been found pathogenic on Spanish peanuts and tobacco. Both crops were stunted in height and reduced in green weight. Peanuts showed considerable root decay but tobacco very little (69).

The genus Paratylenchus (Micoletzky).—The pin nematodes, Paratylenchus spp., like the ring nematodes, have been the object of very little pathological or biological work. They derive their common name from their unusual small size. They pass readily through a 325 mesh screen and probably

have been overlooked in many cases.

Celery is attacked by at least two species of the genus, *Paratylenchus hamatus*, and an undescribed species (75, 76). Populations of *P. hamatus* Thorne and Allen have been found as high as 10,000 per pint of soil in flats of chlorotic celery. Greenhouse tests have confirmed that this nematode is the primary cause of the plant injury.

Weischer (77) found that *Paratylenchus* sp. is a primary parasite of carrot. Because of the staining reaction of infected tissue, he concluded that the

nematode caused chemical as well as mechanical injury.

DISEASE COMPLEX BETWEEN NEMATODES AND OTHER PLANT PATHOGENS

Soil-borne diseases are, in some instances, of complex character. Nematodes frequently are nembers of such complexes and may act as initators, cooperators, synergists, or aggravators of the disorder (4).

VIRUS DISEASES

The importance of nematodes in plant diseases took on a new significance with the important finding of Hewitt et al. (78) that Xiphinema index, Thorne and Allen, transmits the fanleaf virus of grapevines. Hand-picked specimens of X. index from fanleaf-infested soil were washed and placed around the roots of healthy grapes growing in sterilized soil. These healthy plants soon became infected with the virus.

FUNGUS DISEASES

The genus Fusarium (Link ex Fr.).—The relationship between parasitic nematodes and diseases caused by Fusarium has received major attention during recent years. It has been found that Fusarium wilt-resistant cotton varieties will become wilt-susceptible when grown in the presence of large populations of Belonolaimus gracilis (79), Meloidogyne incognita, and M. incognita acrita (80). Similarly, wilt damage was more severe in susceptible cotton if grown in the presence of Rotylenchculus reniformis Linford and Olivera (81). Conversely, the presence of Tylenchorhynchus sp., Trichodorus sp., and Helicotylenchus sp. did not increase the susceptibility of Fusarium-resistant cotton (80).

Similar findings have been related for some other plants. In tomato, the wilt resistance of the variety Chesapeake was broken by the feeding activity of *M. incognita* or *M. hapla* (82). The susceptibility to *Fusarium* wilt was increased in mimosa seedlings by the presence of *M. incognita* or *M. javanica* (83); in alfalfa by the root-knot nematode (84); in banana by *Radopholus similis* (85); in black-eyed bean by *M. javanica* (86); and in tobacco by the tobacco stunt nematode, *Tylenchorhynchus claytoni* (87).

On the other hand, Schindler & Stewart (88) have presented data which indicate that the presence of both Ditylenchus sp. and Fusarium oxysporum f.

dianthi decrease the incidence of wilt symptoms of carnation cuttings, compared to the severity of disease when only the fungus is present.

The genus Phytophthora (de Bary).—The fungus P. parasitica Dast var. nicotianae (B. de Haan) Tucker in the presence of either Meliodogyne inincognita or M. javanica caused the tobacco varieties Dixie Bright 101 and 102 to wilt 75 to 100 per cent within three weeks. The fungus alone caused 0 to 30 per cent wilting (89). Field observations by Moore et al. (90) show the same effect with Meloidogyne spp., Pratylenchus spp. and, to a lesser degree, Tylenchorhynchus claytoni. An attempt to breed tobacco varieties resistant to both nematodes and the fungus, met with some success. The varieties selected developed normally with increases in yield of 14 to 30 per cent over Dixie Bright 101.

The genus Rhizoctonia DC. ex Fr.—The combined effect of Pratylenchus minyus Sher and Allen and the fungus R. solani Kuhn on winter wheat was studied by Mountain & Benedict (91). The growth of winter wheat in the presence of either pathogen alone was almost twice that of wheat grown where both pathogens were present. Reynolds & Hanson (92) noted that the same fungus on cotton was more severe in the presence of a high population of the root-knot nematode Meloidogyne incognita acrita.

BACTERIAL DISEASES

M. incognita acrita and Pseudomonas solanacearum E.F.Sm. were tested alone and together on the tobacco variety Dixie Bright 101 (93). The two pathogens together caused wilt symptoms to develop more rapidly and more severely. The same rapidity and severity of symptoms could be demonstrated by the addition of bacteria to mechanically injured roots. Stewart & Schindler have shown the same effect on carnations with the bacterium Pseudomonas caryophylli Burk. in combination with Meloidogyne hapla, M. incognita, M. incognita acrita, M. arenaria, Helicotylenchus nanus, and Xiphenema diversicaudatum. However, the addition of Ditylenchus sp. with the bacterium resulted in less disease (94).

Crosse & Pitcher (95) have found a definite interrelationship existing between the bacterium Corynebacterium fascians, and either one or both of the nematodes Aphelenchoides ritzema-bosi (Schwartz) Steiner or A. fragariae (Ritzema Bos) Christie. The two organisms produce the cauliflower disease of strawberry only when in combination. No abnormalities appear in the presence of either the bacterium or nematode separately.

NUTRITIONAL DEFICIENCIES

Nematode attack on plant roots results in a restricted root system, necrotic lesion, scar tissue, and some nematodes cause chemical changes in the plant. These factors may result in a less efficient utilization of available soil nutrients by the plant. This effect is expressed as decreased yields and sometimes as mineral deficiency symptoms. Corn infested with *Pratylenchus zeae*

Graham developed severe magnesium deficiency symptoms while noninfested corn remained healthy (96). Bean plants infected with root-knot nematode contained less nitrogen, phosphorus, calcium, and magnesium than noninfected plants (97). Bean plants infested with *Heterodera gottingiana* Libscher had a reduction of nitrogen nodules, crude protein, and chlorophyll (98).

CONTROL

Many different methods have been proposed for controlling nematodes. These may be classified as methods for (a) artificially disturbing the life cycle, for example, by hatching stimulants or by crop rotation; (b) use of predators; (c) improving the natural resistance of plants by breeding; (d) killing nematodes in plant tissue by hot water treatments; and (e) killing the nematodes with toxic chemcials either in the soil or in the plant.

Hatching stimulants.—Studies by British workers (99, 100, 101) on hatching stimulants have shown that certain plant diffusates will cause nematode eggs to hatch. Although several aspects of the subject have been developed, no practical biological control has been achieved to date.

Crop rotation.—As host ranges have been established for various plant parasitic nematodes, recommendations have been made for control by crop rotation. However, with the increased tempo of our present agricultural practices, effective crop rotation is sometimes not economical, and other control measures must be utilized.

Predators.—Many reports have been made on various organisms which are predacious to nematodes (102, 103, 104). These have been confined mostly to simple observations, with little work on the utilization of predators in nematode control. Most nematologists agree that this type of control is not promising.

Resistant plants.—Breeding plants resistant to nematode attack has met with considerable success. New oat and alfalfa varieties have been bred for resistance to Ditylenchus dipsaci (105, 106). Certain varieties of potato have been found resistant to Heterodera rostochiensis (107, 108, 109). Six varieties of rice have been found resistant to Aphelenchoides besseyi Christie, the nematode causing white tip of rice (110). Several pepper, tomato, plum, and sweet potato varieties have been found resistant to one or more species of root-knot nematodes (111 to 114).

Hot water treatment of plants.—This process involves determining the correct time and temperature for killing the nematode but not the infected plant. This method of control has been used for many years and the early work has been summarized (1). During recent years, hot water control measures have been worked for nematode-infected strawberry (115, 116), shallots (117), prune and peach trees (118), and citrus (119).

Chemical control.—Soil fumigation for controlling parasitic nematodes has increased beyond all expectations during the past few years. Many new

materials have appeared on the market, mostly of the volatile type. These materials have a sufficiently low vapor pressure to insure ready permeation through the soil. Advantages and disadvantages of these materials have been summarized by Parris (120).

In all aspects of plant disease control, there appears to be increasing emphasis on plant protection by systemic chemicals. Indications that nematodes can be controlled in this manner have already been shown. Vapors from bis-(dimethylamino) phosphorous anhydride are not lethal to Ditylenchus dipsaci in iris bulbs; however, the nematode is killed by ingesting tissue containing the chemical (121). It is not unrealistic to believe that ultimately, treating seeds or young plants with a chemical will protect the plant from nematode attack throughout the growing season.

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NONSPECIFIC RESISTANCE TO INFECTIONS^{1,2}

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INTRODUCTION

Most animal species show a high degree of nonspecific resistance to infection by many potential pathogens which are found in abundance in their surroundings or which may lead a commensal life in their interior cavities. This resistance is dependent upon a complex equilibrium between many constitutive as well as adaptive defense mechanisms which may be different for each host or even for each anatomical site of infection, and for different pathogens. The measurement of the activity of the different defense mechanisms and evaluation of their relative importance in resistance to infection present many difficulties and these difficulties have been responsible for a long neglect of the subject. Recently, however, the attention of many workers has been refocussed on the field of nonspecific resistance. This intensive study, reflected in the large number of publications and reviews which have appeared in the past few years (1 to 8), has been based mainly on the use of agents which modify the course of infectious processes, the investigation of correlations between states of resistance and the activity of various known defense mechanisms, and the study of in vitro antimicrobial activities of plasma and tissue factors. Much important information on various potential defense mechanisms has been gained in this way. However, the ultimate goal of such work, namely, the integration of our knowledge of the different single factors related to resistance into a complete picture of the defense mechanism of the host as a whole, still appears to be some way off.

Modification of Resistance to Infection by the Administration of LPS

Many experimental procedures have been developed to modify the course of infectious processes. Some serve to promote infection [Olitzki (9, 10)], while others induce a state of increased host resistance towards a variety of pathogens. Increase in nonspecific resistance following injection of various agents has been reported often since the end of the last century and the name "proimmunity" has been given to this phenomenon. The early literature on this subject has been reviewed by Brandis (11). Injections of living

¹ The survey of the literature pertaining to this review was concluded in November, 1958. However, the latest numbers of certain journals were at that time not available to the reviewer.

³ The following abbreviations will be used: EDC (endotoxin detoxifying component); LPS (lipopolysaccharide); RIA (resistance-increasing activity); RES (reticuloendothelial system).

(11) or killed Gram-negative bacteria (12) were found to be among the most active factors inducing "proimmunity" and much recent work has been done to elucidate the nature of the substances in these bacteria which activate resistance. Rowley showed that cell wall fragments of Gram-negative organisms induced increased resistance (13). Further tests with more highly purified cell fractions led to the demonstration that the LPS was the

active principle (14).

This basic finding that highly purified bacterial LPS can induce increased nonspecific resistance has been widely confirmed (15 to 18), and has recently been extended to LPS preparations of mammalian origin (19). This latter discovery may be of importance, since liberated host LPS may play a part in maintaining nonspecific resistance. Degradation of LPS was found by Westphal and his group (20, 21) to yield a subunit, named by them lipide A, which exhibited most of the biological activities of the whole molecule (21, 22). One of the biological activities of the subunit was the induction of increased resistance (16, 21, 23, 24, 25). The protective effect of LPS against levan-enhanced infections with Micrococcus pyogenes and Salmonella typhosa in mice (17, 26), was also produced by the lipide A compound (27). Lipide A, on a weight-for-weight basis, is less active than the original LPS, but measurements of the specific activity of lipide A are beset by difficulties because of its hydrophobic character. Special methods of dispersion in aqueous media have to be employed. With regard to the activity of lipide A, it is of interest that a synthetic cephalin has also been found to induce increased resistance (16, 23).

The question of whether or not purified LPS preparations derived from different Gram-negative organisms are equally active has been carefully investigated by Hurni and his group (16, 23, 24). Lipopolysaccharide obtained in an identical manner from different bacteria produced a biphasic change in resistance, irrespective of its origin, but the degree of activity varied greatly with the source. The equally important problem of how different routes of application affected the RIA of LPS was also studied by this group. LPS administered by the intraperitoneal, intravenous, and subcutaneous routes showed a similar degree of activity, but the appearance of increased resistance was delayed after subcutaneous administration.

The spectrum of resistance induced by purified LPS or cellular components of Gram-negative organisms in various hosts, has been shown to be very broad; it includes resistance to Gram-negative and Gram-positive bacterial infections as well as to infections with viruses. Increased resistance has been induced against Escherichia coli (14, 16, 19), Salmonella typhosa (12, 17, 19), Proteus vulgaris, Salmonella abortus equi (24), Pasteurella tularensis (28, 29), Brucella melitensis (30), Klebsiella pneumoniae (18), Mycobacterium tuberculosis, Mycobacterium fortuitum (23, 31 to 33), Micrococcus pyogenes (17, 32, 34), Streptococcus sp., and pneumococcus sp. (35).

The resistance to certain virus infections such as Columbia-SK. encephalomyelitis and ectromelia is also increased after pretreatment with LPS

(36, 37). In the case of the influenza virus the effect of LPS on resistance is more complex. Animals pretreated with LPS have an increased neutralizing activity of the serum towards egg-adapted virus (38) and show an increased resistance to the toxic effects of large doses of influenza virus (39, 40). Resistance to infection, however, is markedly lowered and, in the lungs of LPS-treated animals, the virus multiplies more readily (41). It has been suggested by these authors that the increased virus-neutralizing activity of the serum (41) may play a part in LPS-induced resistance only in those virus infections which undergo stages of viremia.

Increase in nonspecific resistance could be produced not only by LPS from Gram-negative organisms but also by whole killed cells of *M. tuberculosis* (BCG) and *M. fortuitum* or their extracts (34, 42, 43). The nature of the

active sustances from mycobacteria has not yet been elucidated.

Substances such as zymosan (18, 44), colloidal sulfur (18), thorium dioxide (45), and fine crystalline silica dust (46, 47) have also been found to have some RIA towards experimental infections. In the case of colloidal silica it has been suggested that some relation may exist between increased resistance and the appearance of a humoral factor, somewhat similar to properdin and conglutinin though not identical with them. Lycopene has been shown to increase the resistance of mice to K. pneumoniae infections. The natural and synthetic preparations of the "all trans" isomer of lycopene have been found to be more active than the cis compounds (48).

While some colloids other than LPS can thus induce increase in resistance, most of them are effective only in doses exceeding those of LPS by a factor of 1000 or more. Different mechanisms of action may therefore underlie the activity of LPS and the activity of most other colloids. The possibility has not been excluded that such colloids may cause the release of some endogenous host LPS shown to be ubiquitous in animal cells (19, 49, 60), or that contamination by small amounts of highly active LPS may be responsible for their RIA. Indeed, the presence of trace amounts of an LPS-like contaminant in certain preparations of native levan of Aerobacter levanicum has recently been shown to be responsible for their RIA (17, 26). The native levan freed of this contaminant was devoid of any detectable RIA but retained its infection-promoting activity, its suppressor effect on acute inflammation, and its necrosis-promoting activity in dermal staphylococcal lesions.

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The striking modification in resistance produced by minute amounts of LPS has stimulated attempts to find the mechanisms by which it affects host reaction to infection. These studies clearly demonstrate that many apparently unrelated biological systems, both humoral and cellular, are affected by injection of LPS. Among these are mobile phagocytes (17, 51), the RES (44), bactericidal serum factors (16), serum lysozyme (52), an inhibitor of hyaluronidase (53), the lipemic clearing system (54), activation of fibrinolysis (55, 56) and inhibition of allergic reactions (57). The change in activity of many of these factors following injection of LPS showed a correlation with the biphasic change in the resistance of the host. Before it was

realized that many factors are affected by LPS treatment, certain correlations between changes in resistance and activity of single antibacterial agents were interpreted as furnishing proof that a certain agent is mainly responsible for the increase in resistance. Since many factors are affected in a similar manner, however, correlation between changes in resistance and activity of any single factor can no longer be sufficient to prove a casual relationship.

Among the first findings revealed by the study of the RIA of LPS was the correlation between change in resistance and the bactericidal activity of the serum towards certain Gram-negative organisms (14, 16, 24). At about the same time, the discovery of a new serum bactericide, properdin, was quickly followed by evidence that changes in properdin level in the plasma of humans (58) and of animals (15, 28, 59) parallelled the bactericidal activity of the serum. This finding suggested a central role for properdin in the LPSinduced increase of resistance. This view was further substantiated by findings that the increased resistance was only effective against properdin-sensitive organisms. The initial suppression and subsequent increase in resistance were explained respectively by depletion of properdin as a result of its combination with LPS and its subsequent overproduction by the host. However, further investigations have shown that the properdin system alone cannot be responsible for the increase in resistance. Alterations in the stage of resistance to Gram-negative bacteria cannot always be accounted for by the properdin level measured in the plasma (45), and the resistance induced by LPS to infections by certain Gram-positive organisms (17, 26, 32, 34) which are resistant to properdin, must clearly be caused by some factors other than properdin.

Parallel to the studies on changes in activity of humoral factors following LPS treatment, it was demonstrated that administration of LPS greatly altered the activity of cellular defense mechansims. The phagocytic activity of both the RES and of mobile phagocytes has been shown to be stimulated. In mice and rabbits pretreated with either killed Gram-negative organisms or with purified LPS, a markedly enhanced clearance of colloidal carbon particles by the RES followed a short period of decreased activity (60). A similar biphasic change in the phagocytic response of the RES after LPS treatment was demonstrated by studies on the kinetics of the removal of radioactive LPS (45, 61) or labelled chromium phosphate (62). The increased activity of the RES was found to be caused by a transitory increase in activity per unit weight of the RE tissue and to a more lasting increase in size of the liver and spleen (44). The "lipide A" component of LPS behaved in a manner identical with that of the whole molecule by stimulating the phagocytic activity of the RES and by being taken up rapidly by the latter (45).

Boehme & Dubos (32) studied the correlation between the increased resistance of mice to *Mycobacterium fortuitum* and the phagocytic activity of the RES by measuring the rate of clearance of carbon particles from the

blood. The effect on resistance was found to last for several weeks, while the accelerated clearance of carbon was of relatively short duration. However, if LPS-treated animals were infected with *M. fortuitum* the increase in clearance rate was much more pronounced and more prolonged.

Recently, Benacerraf and his group have succeeded in showing, by the use of 32 P-labelled *E. coli* (63) and *M. pyogenes* (64), that LPS treatment stimulates not only the proliferation and activity of the RES but also the formation of a humoral factor essential for the rapid digestion of the phagocytosed bacteria inside the RES cells. This humoral factor could not be absorbed by zymosan and was not inactivated at 56° C.; it differed in these respects from the properdinlike opsonin described by Nanni (65). The recent findings reported by Rowley (66) and by Slopek and his collaborators (67) that a relatively heat-stable serum factor is necessary for the effective intracellular disposal of phagocytosed bacteria, may be relevant in this connection. Evidence that opsonic activity, as measured with polymorphonuclear leucocytes, could not be equated with properdin was provided by Tullis & Surgenor (68), by Rutenburg & Fine (69), and by Willers (70).

An important advance in clarifying the nature of normal serum opsonins has recently been made by Howard & Wardlaw (71). Using perfused isolated rat livers to measure uptake of viable bacteria by the RES, they showed that at least two factors were involved. One factor was a specific relatively heat-stable antibody not requiring complement, the second a heat-labile comple-

ment-requiring substance, possibly identical with properdin.

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Treatment with killed Gram-negative bacteria or BCG led to the formation of greatly increased amounts of yet another nonspecific serum factor (42, 72) which protects immune monocytes against destruction by engulfed virulent *Brucella melitensis* or *Mycobacterium tuberculosis*. This factor was unlike antibody since it could not be removed from sera by absorption by the respective bacteria.

The marked change in the number of circulating leucocytes brought about by agents modifying resistance has long suggested a possible role for the mobile phagocytes in this modification (6). In addition to the quantitative changes in the white cell count, Fleck et al. (73, 74, 75) have shown that marked qualitative changes in the properties of leucocytes may be caused either by injection of a variety of substances which can induce increased resistance, or by conditions of disease and stress. These leucocytes are more adhesive; they are rich in glycogenlike material and they have intense phagocytic activity; they were therefore named "leukergic" leucocytes.

In a similar way, changes in the blood leucocytes, as judged by a marked increase in iodophilic intracellular substances (76) or in change of their surface charge (77), were induced by purified bacterial LPS. An increase in the speed of migration of human granulocytes was also observed following the injection of LPS (78). In addition, a significantly increased intracellular digestion of chicken erythrocytes by peritoneal phagocytes of mice, was

shown to follow the injection of "piromen" a commercial polysaccharide preparation from Pseudomonas (79). Direct leucotactic as well as mobility-enhancing effects were brought about *in vitro* by LPS from Gram-negative organisms (51, 63, 80) or its "lipide A" component (22), while polysaccharides from Gram-positive bacteria were found to be inactive in this respect (80).

Evidence, in vivo, for the existence of an LPS-induced effect on mobile leucocytes has been obtained from experiments with levan-enhanced infections. Pretreatment of rabbits with LPS rendered these animals refractory to the spreading dermonecrosis induced by Micrococcus pyogenes or certain other bacteria (17, 26). Whereas, in "levanized" animals all elements of the inflammatory response including diapedesis were suppressed, in animals pretreated with LPS, active diapedesis occurred at the sites of the lesion in spite of the levan block. As the protective mechanism in these dermal infections is likely to be operative at the site of the lesion, the finding that LPS causes mobilization and local concentration of leucocytes may provide a possible explanation for the mechanism underlying the increased resistance in this system.

The ability of LPS to increase resistance to a wide range of infections has naturally raised the question whether or not it may be of clinical value. Its therapeutic use may, however, be hampered by the initial depression of resistance (negative phase) brought about by LPS treatment. Preliminary experimental evidence indicates that the negative phase may possibly be eliminated by the use of either the intracutaneous route for injecting LPS (16, 23, 44), or by injection of nondispersed "lipide A" (81). Alternately, the injection of small doses of LPS prior to the effective larger dose was shown to eliminate the negative phase.

THE ENDOTOXIN MODIFYING ACTIVITY OF SERUM AND PLASMA

In view of the striking effect of LPS on resistance to infection, any processes which modify it in vivo so that it can no longer act in stimulating defense mechanisms must be of importance. Since the pathophysiology of many diseases produced by Gram-negative organisms depends on the toxic effects of bacterial LPS, any modification of LPS leading to its detoxification could be, in itself, a potent defense.

By the interaction of LPS with serum or plasma *in vitro*, changes in the biological activities of LPS have been obtained by many investigators. The results obtained in the different investigations are difficult to compare because experimental conditions varied in many important respects. The source and method of preparation of LPS, the kind of anticoagulant added to plasma or serum, the criteria and assay systems used for testing LPS inactivation are among the factors which have varied. In some cases, contradictory results have, in fact, been obtained. The position is further complicated by the probable existence in plasma of several factors modifying LPS.

Among the biological properties of LPS which can be modified by serum or plasma, changes in pyrogenicity have attracted special interest. Although earlier findings indicated that treatment of LPS with serum could lead both to augmentation and to loss of pyrogenic activity (82, 83, 84), it has recently been shown (85, 86) that the increase and decrease of pyrogenicity are separable functions. In fact, the augmenting effect appears, after short contact, between LPS and serum, and does not appear to be brought about by an enzymatic process. The loss of pyrogenicity, on the other hand, requires prolonged incubation, and the serum factor responsible for this effect has enzymelike properties, and can be absorbed by zymosan. The factor destroying the pyrogenic activity of LPS has been found in the sera of man (87), of horses (83, 87), and of dogs (83). Using the horse as the experimental animal (88), Westphal and his group confirmed these results (89), and showed that the pyrogenic and leucopenic effects of "lipide A," are also abolished by horse serum and plasma (90).

Realizing the complexity of the subject, Landy et al. (91, 92, 93) embarked on a series of careful tests of the suitability of different biological activities as quantitative indicators for endotoxin inactivation. Induction of damage in tumor tissue in mice of known genetic constitution was chosen in preference to other criteria because of its sensitivity and reproducibility. With this technique, these authors were able to show that citrated plasma of many animal species quickly detoxified LPS. On the other hand, sera from the same species (with the exception of the rat and some human sera) were found to be much less active, the addition of citrate to these sera resulting in greatly increased activity. The inhibition of the endotoxin detoxifying component (EDC) of calcium was shown to account, in part, for these results, and presumably this inhibition also explains many of the discordant findings in the literature where serum or plasma with and without the addition of calcium-binding agents have been used.

The requirement for anions, the inhibition by calcium, and the fact that magnesium is not needed for activity clearly distinguish EDC from properdin.

Another property of LPS which is modified by incubation with serum or plasma is the antigenic behavior as tested by the reduction in antibody precipitable material (94) or by the change in precipitation zones in gel diffusion tests with antibody (95). The agent which modifies the immunological properties of LPS can be precipitated from the serum by 50 per cent ammonium sulfate and does not require magnesium for its activity, nor is it absorbed by zymosan (94).

The complexity of the interaction of serum factors with LPS as illustrated by the findings (96, 97) that the lethal action of crude endotoxin, but not of highly purified LPS, may be destroyed by human, rabbit, and rat plasma. The factor responsible for the attenuation of this property of crude endotoxin is relatively heat-stable, different from both properdin and antibody, and is confined to the serum protein fraction II+III of Cohn (98).

Many of the properties of the LPS-modifying systems discussed above suggest that they might be enzymatic in nature. Earlier observations by Pillemer & Landy (99) which showed that incubation of LPS with human serum resulted in increased dispersion, decrease in turbidity, and reduction of the sedimentation rate suggest an enzymatic depolymerizing activity of the serum. Clear evidence for the presence of an enzyme in serum capable of splitting inorganic phosphate from purified LPS has been presented by Rowley and his collaborators (100, 101), using 32P-labelled LPS. This enzyme requires for its action the presence of magnesium ions, is capable of attacking LPS from a wide variety of bacterial sources, and splits off in amounts up to 25 per cent of the phosphate found in the LPS. Experiments attempting to elucidate the relation between dephosphorylation and loss of biological activities of LPS have not yet been described. Interpretation of such experiments in which serum is the source of phosphatase might be difficult, since serum may contain many other factors with LPS-detoxifying activity. The use of purified calf intestinal phosphatase, shown to be similar in its activity to the serum phosphatase (101), offers a promising approach to the solution of this difficulty. It is already clear that the serum phosphatase activity is not identical with properdin, since it has been possible to remove properdin activity from sera without affecting their phosphatase activity. The requirement for magnesium and the lack of inhibition by calcium differentiates the phosphatase from the EDC of Landy and his collaborators (92, 93).

Lysis of Cells by Serum Factors

The ability of sera to lyse a wide variety of bacterial cells, certain pathological cells of animal origin [e.g., neoplastic cells (102, 103), the erythrocytes of patients with nocturnal paroxysmal hemoglobinuria (104, 105, 106)], and a number of protozoa (107, 108), as well as their ability to inactivate viruses (109, 110) has been reported by many workers. This wide range of activities involves different systems, which have been admirably discussed in a recent review by Skarnes & Watson (1). Some of these systems may have broad spectra of activity upon a widely distributed substrate in the walls of susceptible cells. The finding that LPS is present in all Gram-negative bacteria and in many animal cells suggested that this substance might possibly be such a substrate. Several different lines of investigation have indeed substantiated this hypothesis. Wedgwood, who investigated a serum factor which causes lysis of Gram-negative organisms resistant to properdin, showed that many of its properties were identical with those of the endotoxin detoxifying component described by Landy and his colleagues (92, 93). Properties common to both systems were inhibition by calcium ions, absorption by zymosan and LPS, and lack of requirement for Mg++ and for the first and fourth components of complement (111). Experiments indicated that LPS may indeed be the substrate of the bactericidal serum factors since lytic activity against Shigella dysenteriae (112) or S. typhosa (113) was inhibited by the homologous O-antibodies. Additional evidence that the O-antigens are the site of action of the serum bactericidal activity was produced by Muschel, Chamberlin & Osawa (114), who demonstrated a correlation between O-inagglutinability due to Vi antigen content and resistance to the bactericidal effect of normal

human, guinea pig, or rabbit serum.

Light has been shed on the mode of action of some serum bacteriolysins by the demonstration that cells of various Gram-negative organisms are converted to protoplastlike bodies by fresh normal serum in the presence of stabilizing concentrations of sucrose (115 to 118). Further investigation of the products released from susceptible cells during protoplast formation may lead to the identification of the substrates in the bacterial cell wall attacked by the bacteriolysin. This site of action must be different from that which interacts with penicillin, since protoplasts formed by incubation with serum can be lysed, in the presence of sucrose, by penicillin and those formed under the influence of penicillin can by lysed by the serum bacteriolysin.

The findings of Amano et al. (119) and Inai et al. (120) recently confirmed by Muschel and his co-workers (117, 121) may help to clarify the nature of some serum bacteriolysins. These workers have shown that lysozyme will produce lysis or protoplasts when acting on Gram-negative organisms in the presence of small amounts of the antibody-complement system. The fact that lysozyme was demonstrated by the latter group in purified properdin preparations, suggested that lysozyme may be a possible reactant in the bacteri-

cidal activity, hitherto ascribed to properdin.

The similarity between the serum systems lysing Gram-negative bacteria and those lysing certain animal cells (erythrocytes) has been pointed out by

several investigators (104, 105, 106).

Evidence has been found to lend support to the hypothesis that LPS may be the active substrate not only for serum bacteriolysins but also for the factors attacking animal cells. Intact erythrocytes or stroma when treated by techniques known to extract LPS from Gram-negative bacteria, yield polysaccharides with typical LPS properties (50, 104, 122, 123). Besides the above mentioned pathological cells, normal cells become susceptible to lysis after treatment with tannic acid (105) or after treatment with proteolytic enzymes, protamines, and histones (106). This suggested that the site of action of the lysins had first to be uncovered, and this uncovered substrate could have been LPS. It has also been shown that normal human erythrocytes become sensitive to lysis by serum from heterologous species, after having been coated with purified LPS (124).

THE PROPERDIN SYSTEM

Three comprehensive reviews (1, 2, 7) dealing with antimicrobial serum and tissue factors including properdin have recently appeared, and special reviews have been published on the properdin system (125 to 130). The wide

range of activity of the properdin system *in vitro* and the correlation between properdin levels of plasma and resistance have stimulated numerous investigations to elucidate the importance of properdin in resistance.

The nature of properdin.—The special cofactor requirements of the properdin system, the lack of specificity of its action and the selective inactivation of the third component of complement by the properdin-zymosan complex led the discoverers of properdin to suggest that it is a unique entity with no relation to antibody.

This early view has recently been modified and the suggestion has been made (126, 129) that properdin may possibly be a special case of normal antibody which has many analogies to certain hemagglutinins. In particular, Skarnes & Watson (1) have stressed the numerous similarities between properdin and known antibodylike factors. Similar evidence has been brought by the experiments of Blattberg, who has shown that immunization with zymosan results in the increase of properdinlike substances in the serum (131, 132). Further evidence supporting the hypothesis that the properdin system can be fully explained in terms of classical, normally occurring antibody, has been put forward recently by Nelson (133). He suggested that the striking lack of specificity in the reactions of the properdin system was based upon the widespread occurrence of the responsible antigen rather than to a unique nonspecificity of the antibody, and showed that most sera from normal humans are capable of sensitizing zymosan to react in immune adherence, immune aggregation, and complement fixation. Even highly purified properdin preparations were shown by him to contain components capable of agglutinating zymosan.

The work of Turk (134) has shown that the interpretation of the phenomena of immune adherence and immune aggregation may be more complex than hitherto thought. At least two separable active principles, different in properties, were demonstrated to be involved in these reactions and their effects were often superimposed on each other. The one reacted specifically with bacteria or particulate polysaccharides and was indeed similar to classical antibodies, the other, of broad specificity, showed many similarites to the properdin system. Proof that conglutinin of zymosan and properdin are separable factors and require different cationic cofactors has also been reported by Leon (135).

Purification and properties of properdin.—Properdin can be purified by absorption on zymosan and consequent dissociation of the properdin-zymosan complex, to give 10 to 20 per cent of the original properdin with several thousandfold purification (136). Certain modifications of this procedure suitable for large scale production have been introduced recently (137 to 140). Isolation is also possible from fraction I of serum (Cohn's method) without the use of zymosan (141, 142). This is of special importance, since even the purest properdin samples obtained by absorption on zymosan still contain traces of the polysaccharide.

Discrepancies found in the size of the properdin molecule have been resolved by elegant experiments (143), showing that properdin is a macroglobulinlike substance which has a sedimentation constant of 16 to 25 S. In the presence of mercaptans the large units dissociate into smaller, homogeneous fragments, the sedimentation constants of which are 6 S, and these smaller units can reaggregate in the presence of O₂. On the basis of the inhibitory effect of monoiodoacetate on reaggregation, the 6 S units were suggested to contain freely reacting sulphydryl groups which form intermolecular disulphide bridges in the parent macroglobulin.

Properdin has antigenic properties which have made it possible to obtain specific antihuman properdin antibody in rabbits (144, 145). Immunoelectrophoretic analysis of properdin has shown that it has the proper-

ties of a β -globulin (146, 147).

Studies on the cofactor requirements of the properdin system have shown that certain new factors are involved. Among these a hydrazine sensitive factor, necessary for the inactivation of the third component of complement (C'3) by the properdin-zymosan (PZ) complex has recently been described (148). This factor, although similar to the fourth component of complement, differs from it in many respects. The complexity of the interaction of properdin and zymosan is indicated by the findings of Leon (149) who has carefully investigated the kinetics of this reaction. He suggested the presence of at least two consecutive reactions prior to the formation of the complex which inactivates C'3. It has further been shown (150) that the reactions which lead to the formation of the PZ complex and those of the inactivation of C'3 by this complex are affected in opposite directions by dilution of the serum. Contrary to earlier claims it was demonstrated by Leon that Mg++ is not required for the inactivation of C'3 by the PZ complex. Differences in the cation cofactor requirements of properdin in its different interactions have also been shown by Wardlaw, Blum & Pillemer (151).

Assay of properdin.—The development of reproducible and simpler properdin assay systems has occupied the attention of many workers. The lack of such a system has been an important factor hindering progress in this field.

Among the new developments in assay methods, modifications (152 to 156) of the zymosan-complement technique of Pillemer et al. (157) have been prominent. Some of these modifications contain corrections for the C'3 content of the sera under test. Others introduced the use of inulin instead of zymosan (158, 159). Recently, the removal of properdin-binding inhibitors from the serum prior to the inactivation of C'3 by the PZ complex has been proposed (70, 160, 161, 162). The use of this procedure permits the estimation of "total" properdin, while the earlier methods estimate the "available" properdin only. A comparatively simple and reproducible method for the estimation of properdin using inactivation of the T2 r+coli phage has recently been described (110, 163). However, quantitative agreement has not always been found between the phage inactivation

(162, 164) or the neutralization of Newcastle virus (70) and the estimation of properdin by the zymosan test. This has been explained (163) as perhaps resulting from the requirement of different cofactors in the different systems. An additional difficulty in the interpretation of the phage inactivation test was demonstrated by Cowan (165), who compared the inactivation of three different phages (T3, T6, T7) by different sera. The ratio of the activities of each serum against the three phages was determined and was found to be different for each serum. Since this could not be caused by varying susceptibilities of the phages to a single neutralizing substance, Cowan concluded that with each phage, and possibly with each indicator system used for assay, different substances, all capable of combining with zymosan, might be measured.

Evaluation of the role of properdin in nonspecific resistance.—Much evidence has been published during the past few years to indicate that properdin plays an important part in nonspecific resistance. Some of this evidence is mentioned in other sections of this review. Because of its complexity, much of the evidence linking the properdin system to states of resistance is of an indirect nature and is based to a large extent on correlations. Recently, the role of properdin in defense has been questioned by various workers on the basis of new experimental evidence. A re-evaluation of the whole problem is thus indicated.

Various authors have inferred, from the protective effects demonstrated after treatment with purified properdin preparations, that properdin is active in defense against infection. However, the earlier results (166, 167) are open to criticism since zymosan and possibly other impurities were present in the properdin preparations used. In a more recent investigation wherein protection was demonstrated against *Klebsiella* infection after treatment with zymosan-free properdin (168), equal protection was obtained with heat inactivated (100° 30 min.) properdin solutions.

In many investigations zymosan has been used specifically to absorb properdin. The removal of a certain activity, whether in vivo or in vitro, by treatment with zymosan has often been used as the main proof that properdin is the responsible agent for that activity. Such interpretations may, however, be misleading sinze zymosan is a very heterogeneous substance (70) and cannot be considered as a specific absorbing agent for properdin. It has been shown that a mixture of many proteins is absorbed from the serum by zymosan (169) among which are lysozyme (117, 170); bactericidins against Bacillus anthracis (171); Micrococcus pyogenes and Bacillus subtilis (170); a phagocytosis-promoting factor (68); serum phosphatase (101); and normal antibody (133, 134, 135).

While in the early investigations the correlation between serum bactericidal or viricidal activity and properdin levels appeared to be very close recent findings have shown that in some cases such correlation is completely lacking (70, 162, 164, 169, 172). Willers (70) has also produced evidence which he interprets as showing that properdin cannot be involved in

the bactericidal and viricidal activities of sera against organisms considered to be sensitive to properdin. His proof consisted of (a) exhaustive absorption of properdin from sera did not remove the bactericidal or viricidal properties and (b) the bactericidal activity of properdin depleted sera RP_b (which had also been freed of bacteriocidal activity) could not be restored by the addition of purified properdin even in the presence of all the known cofactors of the properdin system. It must, however, be borne in mind that while (a) clearly shows that factors other than properdin can be active it does not exclude the activity of properdin, while the evidence in (b) could be explained by the absence of a yet unknown essential cofactor of the properdin system. It may be pointed out in this connection that difficulties have been experienced in restoring yet another activity sometimes claimed for properdin, namely opsonic activity upon the addition of purified properdin to RP_b sera (71).

The specific role of properdin has been stressed by the fact that antiproperdin antibody neutralizes the bactericidal, antiviral, and cytolytic (144, 173, 237) properties of serum. These results however, have to be interpreted with caution, since the purified properdin preparation used as an antigen may have contained other active substances. In fact, lysozyme activity was found in highly purified properdin preparations obtained with (117) or without (52) the use of zymosan or other polysaccharides. It has also been shown (117) that antihuman properdin sera precipitated crystalline egg-white lysozyme. Phosphatase activity has also been shown to be present as a contaminant in all purified properdin preparations tested (101).

The role of properdin in neoplastic growth.—The cytotoxic effect of normal serum in vitro (102, 103) against certain cancer cells in tissue culture (174) or in vivo against some neoplastic growths (175) has often been described. Several investigators using different experimental approaches have suggested that the properdin system is an important factor involved in the cytotoxic activity of serum. This system was further proposed to be involved in resistance to heterotransplantation and in the active regression of transplantable tumors. The evidence which led to this hypothesis may be summarized as follows: (a) A correlation has been shown to exist between clinical human cancer (176, 177) or experimental cancer (178, 179, 180), and low plasma levels of properdin. (b) Treatments which induced markedly increased properdin level, such as the injection of LPS, were shown to increase significantly the spontaneous regression of transplanted Yoshida Sarcoma in rats (181) and of some lymphomas and sarcomas in human patients (182, 183). (c) A reduction in resistance to heterotransplantation has been demonstrated to follow the injection of high doses of zymosan or exposure to ionizing radiation (180, 184, 185, 186), treatments known to lower properding levels, whereas small doses of zymosan which raise the properdin level were shown to increase regression of Sarcoma 118 in mice (184).

The liberation of increased amounts of α -2 globulin (183, 187), heparin (188), or endogenous LPS (19) has been shown to occur in conditions of

tissue damage, shock, or stress. Such inactivation of properdin by α -2 globulin has, in fact, been demonstrated (189) and could be an adequate explanation for the low properdin levels found in clinical or experimental cancer. In addition, evidence for the direct consumption of properdin by necrotizing tissue of a rat sarcoma has been presented (190). Low properdin levels thus may be an outcome of the development of cancer, rather than a factor of predisposition towards cancer.

Detailed studies of the lytic activity of serum against certain tumor cells in vitro, previously believed to be caused by the properdin system, have shown that the full activity could also be demonstrated in sera exhaustively depleted of properdin or of some of its cofactors (178, 191). The humoral factor involved must therefore be different from properdin and its exact

nature still remains to be elucidated.

Thus, while a possible function for properdin in protection against neoplastic diseases cannot be finally excluded, no proof of any important role of properdin in this connection has yet been given. The large scale production of properdin, recently initiated by the Sloan Kettering Foundation (126) for extensive clinical tests of the therapeutic value of properdin against different forms of cancer, may finally clarify this question. It may be pointed out, however, that any therapeutic use of properdin will have to face a number of problems. Among these are the antigenicity of properdin (144) which might allow its use from a homologous source only, its occurrence in sera in trace amounts, its extremely quick turnover in the host (168, 192), and the presence of endogenous inhibitors released in great amounts in conditions of tissue damage (183, 188).

THE NONSPECIFIC DEFENSE MECHANISMS IN VARIOUS PATHOLOGICAL CONDITIONS

Several pathological conditions induced by chemical or physical means are known to lower resistance to infection and have been used in the study of defense mechanisms.

Irreversible hemorrhagic shock.—Fine and his collaborators have found that in hemorrhagic shock animals show a markedly lowered resistance to infection because of impairment of the humoral and cellular mechanisms of defense (193). Thus, under conditions of hemorrhagic shock, intravenously injected bacteria are incompletely removed from the blood stream of dogs (194), the sensitivity of rabbits to the lethal effect of endotoxin is increased a hundred thousandfold (195), and the phagocytosis-enhancing effect of serum is lowered (194). The plasma of shocked animals possesses leucotoxic effects in vitro (196) and the capacity of such animals to mobilize phagocytes to sites of challenge was found to be impaired (197). While the properdin levels of plasma from dogs in shock were shown to drop progressively to very low levels, evidence was found that this was not the reason for the reduced phagocytic activity (69). Precooling of the animals prior to shock

seemed to protect the normal bactericidal mechanisms of the host (198), and delayed the onset of irreversible changes. On the basis of these findings, the invasion of commensal intestinal organisms into a shocked host was suggested to be the cause of death after shock. The decisive part played by bacterial invasion in the production of irreversible damage in the shocked animal was substantiated by the protective effect of treatment with antibiotics (193). Further evidence for the possible importance of Gram-negative intestinal bacteria in the production of irreversible hemorrhagic shock is the resemblance between the pathophysiology of hemorrhagic shock and the damage caused by endotoxin (199, 200), as well as the demonstration of a lethal (201) endotoxinlike (202) substance in the tissues and plasma of shocked animals. However, recent experiments with germ-free rats (203) have shown that hemorrhagic shock can be induced in the absence of any viable bacteria. These results indicate that bacteria are not an essential requirement for the production of irreversible hemorrhagic shock, but they do not exclude the possibility that bacterial infection may play a part in shock in normal animals or that bacterial products in the food of germ-free rats are involved. The proof of the bacterial origin of the lethal endotoxinlike material found in shocked animals could possibly be achieved by the use of immunological methods making possible the recognition of characteristic deoxysugars in bacterial LPS (204) or by the use of 32P-labelled bacteria.

Experimental liver damage.—The finding that the development of cirrhosis caused by choline deficiency (205) is markedly delayed by antibiotics has suggested that intestinal bacteria are largely responsible for the liver damage. These findings have similarities to those obtained in irreversible hemorrhagic shock. Eger has shown that the resistance of rats to liver damage by injected allyl alcohol is modified by pretreatment with LPS (206), zymosan and dextran (207), or total body irradiation (208). The resistance to liver damage and, similarly, resistance to bacterial invasion induced by these compounds was at first lowered and then increased. The conclusion was drawn that resistance to liver damage by allyl alcohol may be a measure of resistance to bacterial infection and the authors have suggested that properdin might be the responsible factor for both phenomena. This conclusion, however, must be considered as purely hypothetical for the present.

Ionizing radiations.—The susceptibility of different mammalian species to a variety of infective agents is markedly increased following x-ray, γ -ray, and fast neutron radiation. The available evidence suggests that infection is a major cause of mortality in animals exposed to close-to-lethal, whole-body ionizing radiation. Several reviews have appeared covering the earlier literature on this subject (3, 209, 210). A high incidence of bacteremias by intestinal bacteria (211, 212, 213) and an increased establishment of experimentally given microorganisms (211, 214) is found in the irradiated animals. The ability to restrain the multiplication and dissemination of the

bacterial invader is diminished (211). Further proof of the role of infection in irradiation damage has been furnished by the reduction of mortality by treatment with antibiotics (211), and by the relatively high resistance to irradiation of certain germ-free animals (215).

In animals treated with supra-lethal doses of ionizing radiation, antibiotics do not reduce mortality and it may be inferred that factors other

than bacterial invasion are responsible for death (216).

The study of the time relationship between the onset of generalized infection in irradiated animals and the damage caused to the different defense mechanisms has helped to evaluate their relative roles in resistance. Bacteremias are usually observed during the second week after close-to-lethal doses of radiation, whereas the activity of many of the known defense mechanisms is markedly decreased at a much earlier stage (211, 217, 218). Among the defense mechanisms affected within the first days, long before bacterial invasion becomes evident, are the mobile leucocytes, and the intestinal mucosa (211). An interesting fact which has emerged from this work is that even in normal animals, the intestinal mucosa is not an effective barrier to invasion (219), and after irradiation its permeability is not increased (211, 218). Prolonged treatment with low doses of γ -radiation quickly induces severe leucopenia, but resistance to experimental infection remains unchanged for a long time (211, 212, 220).

The finding of some correlation between lowered resistance in postirradiative conditions and low properdin levels (221, 222, 223) and the protective effects of treatment with zymosan (167) and purified properdin resistance to (166, 167), has led certain workers to ascribe importance to properdin in irradiation damage. These experiments were, however, shown to be difficult to reproduce (121, 224). Recent investigations have also shown that the low properdin levels in irradiated animals are likely to be caused by the liberation of the endogenous substances which bind properdin, such as those liberated in other conditions involving extensive tissue damage (183). The rate of properdin biosynthesis as measured by the incorporation of ¹⁴C-labelled leucine (223) is not altered.

Analysis of the contributory roles of the different defense mechanisms has led many investigators to conclude that the critical injury sustained in irradiation involves the RES (225). Good correlation was found between the destruction of the RES and time of appearance of bacteremia in irradiated animals (211, 226). The main damage sustained by the RES does not lead to cessation of phagocytosis but to failure to eliminate phagocytosed bacteria (211, 226, 227) or endotoxin (228).

Relation of the inflammatory response to defense.—A fruitful and elegant approach to the study of natural defense mechanisms has been developed by Miles and his co-workers, who have developed techniques for measuring even minute responses of the host in skin infections. After analyzing quantitatively the kinetics of the development of dermal lesions initiated by small

numbers of various bacteria, the authors showed that an early short critical period exists which determines whether the pathogen achieves lodgement in the body (229). Having established the importance of this period, these workers investigated the detailed sequence of vascular events following the infective stimulus. They found no striking inflammatory reaction during the first hour, in spite of the fact that during this time inoculated bacteria were most rapidly rendered ineffective. This and the lack of correlation between capillary permeability as measured by extravasation of colloidal dyes and the extravascular accumulation of blood elements, indicated the importance of a critical re-examination of the relationship of vascular permeability to effective defense against local infection (230). In addition, evidence has been reported which controverts the role of lymphatic blockade as an effective peripheral defense in acute inflammatory lesions (231).

A different approach to the study of the role of inflammation as a defense mechanism was made possible by the discovery of the antiphlogistic activity of neutral high molecular polysaccharides (levans and dextrans). The suppression of inflammation is strikingly shown when "levanized" animals are infected intradermally or treated with chemical irritants and compared to nonlevanized controls (17, 232, 233, 234). Further investigation showed that in animals given levan, extravasation of blood cells (232) and colloids (γ -globulins and trypan blue) (233) is blocked. The hypothesis has been advanced that the infection-promoting activity of the polysaccharides, both against intradermal and intraperitoneal infections (17), may result from their blocking of transfer of antibacterial factors from the vascular pool to the local sites of infection.

This hypothesis was substantiated by experiments in which "levanized mice" were infected intraperitoneally with Salmonella typhosa and the levan block was overcome by direct placement of serum or serum fractions in the site of infection. A high degree of protection was afforded by mouse serum, sera from various other animals, and the III-1 bovine serum fraction of Cohn (27).

The protection afforded by whole plasma as well as by certain purified γ -globulin fractions of serum against *Pseudomonas aeruginosa* infection (235, 236) in mice may also be pertinent in this connection.

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BIOSYNTHESIS OF ANTIBODIES, FACTS AND THEORIES^{1,2}

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Immunology suffers, as do many other branches of science, from an enormous increase in the number of publications, since it is at once a fundamental discipline and a technique which finds its application in many different fields: Medicine (Allergology-Serotherapy-Diagnosis), Biochemistry, Physico-chemistry, Virology, Genetics, Zoology, etc. Biological Abstracts analyzes annually about 10³ publications dealing with general immunology, and it is impossible to refer to all of these papers or, in fact, to read them all, much less to assemble publications which are not abstracted. In view of this situation the reviewer has decided to limit the scope of this paper to a small number of sections which are more or less directly related to the biosynthesis of antibodies at a cellular level.

Immunology has benefited greatly, in recent years, from the discovery of adaptive enzymes in microorganisms and from the recent advances in this field. Unfortunately, the immunologist's material-higher organisms studied for long periods—has compared unfavorably with that available to his colleague in microbiology who studies the metabolism of reasonably homogeneous cell populations for a short period of time. However, the development of cell culture methods is rapidly eliminating this difference. There is little doubt that the knowledge gained in the study of specific synthesis in microorganisms suggests to the immunologist some working hypotheses to be tested in his own field. It must be noted that these hypotheses, as they pertain to antibody production, still await experimental check. The completion of the full sequence of events beginning with stimulation by antigen and ending with antibody production, all within a single clone in vitro, has yet to be accomplished. The major stumbling block to this achievement is a technical one and it is the opinion of the reviewer that efforts should be concentrated on overcoming it.

Recent publications offer a great body of technical data which are useful in approaching this aim, but the theoretical implications of many of these works are not clearly brought to light.

The following terms will be used, in order to avoid confusion, in the

¹ The survey of literature pertaining to this review was concluded in December, 1958.

² The following abbreviations will be used: Ab (antibody); Ag (antigen); BGG (bovine gamma globulin); BSA (bovine serum albumin); DNA (deoxyribonucleic acid); HGG (human gamma globulin); HSA (human serum albumin); RES (reticulo-endothelial system); RNA (ribonucleic acid).

³ I am greatly indebted to Dr. M. B. Groman for his invaluable assistance in writing the English version of this review and for his useful criticisms.

course of this review: immunogenicity: ability of a substance to direct the formation of a specific antibody; antigenicity: ability of a substance to react with an antibody under a given set of conditions. An immunogen is a substance exhibiting immunogenicity and an antigen, a substance exhibiting antigenicity.

FACTORS INFLUENCING IMMUNOGENICITY

Immunogenicity depends upon an interaction between the substance administered and the recipient organism. We will try to distinguish between the two kinds of factors which influence it. (a) Specific: related to the nature and structure of the substance injected. (b) Nonspecific: conditioning the reactivity of the recipient. It is difficult, in some cases, to separate those factors which pertain essentially to the immunogen and those which influence the recipient. For the sake of clarity we will attempt to discuss them separately.

SPECIFIC FACTORS

Immunogenicity is not an abstract notion, as evidenced by its relation to many technical details of immunization, e.g., nature and physical state of the substance, route of injection, species, and physiological state of the recipient, and so on.

It has long been known that a minimum molecular weight is required for immunogenic activity; nevertheless, this minimum differs greatly according to different authors [see Landsteiner (1) pp. 62 and following]. The shape of the molecules may also play a role, as it has been observed with synthetic polypeptides that a branched polypeptide of MW:13,000 is immunogenic [Dellert et al. (2)], and a linear polypeptide at MW:80,000 is nonimmunogenic [Maurer (3)].

Many details of structure of the molecules, such as the absence of aromatic amino acids in gelatin [Neuberger (4)], have been invoked to explain the nonimmunogenicity of certain substances. We must admit that no general rule is yet available which will allow us to predict the immunogenicity of a given substance.

Another aspect of the problem of the true state of the immunogen in the body is the role played by the Ag-Ab complex itself. It has been found that γ -globulins are more immunogenic when given in an Ag-Ab complex (as the Ab part) than when they are given alone [Adler (5)].

Aside from this aspect of the structure of compounds in their relation to their immunogenicity, there is another, more biological aspect, which arises from the concept of "immune tolerance." This concept states that when a substance is present in embryonic life, whether it is "self" or "foreign," it will fail to be immunogenic in adulthood [Burnet & Fenner (6)].

Thus, the development of the host becomes an important factor for the immunogenicity of any substance. In the course of its development, an animal passes through three phases in terms of its reaction to administered

material: a negative phase in which it "learns" to recognize this material as "self"; a neutral phase in which it is indifferent, and a positive phase in which it recognizes this material as "foreign." It is well known that some body substances, e.g., lens proteins, sperm, nerve tissue, thyroglobulins [see Brent & Medawar (7)] are immunogenic if injected in the adult. This property is related to their normal isolation from the Ab-producing centers during the negative phase. This same isolation in the positive phase accounts for the absence of Ab formation in normal adult animals. As this field is reviewed in this same volume we will discuss this problem below only in relation to the question of immunogenicity.

Immune tolerance, if studied by skin graft, manifests itself mainly by an all or none effect (8). However, this tolerance may be only partial as indicated by some results [Wolfe et al. (9)]. The concept of immune tolerance can be generalized on a quantitative basis to state that the more taxonomically foreign a substance is, the more immunogenic it will be [Bussard (10)]; (this is a common feeling among immunologists but the question has never been systematically studied). Support for this idea is given by recent experiments in which rat bone marrow cells were transplanted into x-irradiated recipients [Gengozian & Makinodan (11)]. These cells produced higher yields of agglutinating Ab against more distantly related immunogens than against closely related ones. In the case of insulin, in which we know that the chemical differences between insulins are very small, the low immunogenicity of this protein may be ascribed to immune tolerance, the tolerance of any organism towards its self-substances extending to structurally similar substances.

Although it is true that substances foreign to an organism are more immunogenic than closely related ones, recent studies have emphasized that substances derived from animals of the same species as the recipient, can also exhibit this characteristic. It has been established recently by Oudin (12) that Abs from rabbit A can be immunogenic for rabbit B if they are injected under certain conditions (specific precipitates with Freund's adjuvant). The Abs thus formed against γ -globulins of A react as well with sera of non-immunized rabbits (preimmunization serum of A and others).

The name "allotypes" has been given to the individually different antigenic forms of soluble circulating proteins which were considered, until now, to be identical within a species. Their presence and form are genetically

controlled.

The denaturation of the γ -globulin from A by the Ag-Ab reaction has been invoked to explain its immunogenicity [Milgrom & Dubinski (13)], but this explanation is not necessary as it is possible to immunize a rabbit by the injection of whole serum of another rabbit together with Freund's adjuvant [Dray & Young (14)].

The discovery of "allotypy" enlarges our notion of individual immunological specificity which was restricted, up to now, to cellular antigens. This phenomenon may play a role in transplantation immunity [see review by Brent (15)], as the transplanted cells could elicit a reaction in the host not only by their fixed cellular components but also by the soluble products which they liberate.

Recent discoveries in transplantation immunity also illustrate the role played by the physical state of the immunogen. The factor responsible for transplantation immunity (skin grafts) in the mouse, was thought by Billingham et al. (16) to be related to a deoxyribonucleoprotein. After further studies it now appears that a polysaccharide is implicated [Medawar (17); Billingham et al. (18)]. However, it seems as if the association of this polysaccharide with cellular DNA plays an important role in its immunogenicity since deoxyribonuclease is still capable of destroying immunogenicity.

Factors affecting immunogenicity of a substance can also be studied fruitfully by means of isolated cells. This has been done recently with lymph node cells in vitro [Harris et al. (19)]. These cells were sensitized in vitro with a trypsinized extract from Shigella organisms and later showed Ab formation in vivo, whereas intact bacterial cells were nonimmunogenic towards the lymph node cells. These results lead us to consider the state in which the immunogen is present at the site of Ab synthesis. It is conceivable that this state may be quite different from the state in which the immunogen was injected. It has been recently hypothesized that proteins are hydrolyzed in the spleen before they enter the actual site of Ab production. In fact, a protease extracted from the rabbit spleen [Lapresle & Durieux (20)], distinct from cathepsin, hydrolyzes HSA in vitro. However, material so degraded does not elicit the same specific Ab pattern in agar gel as does native HSA [Lapresle & Durieux (21)], and this appears to contradict the hypothesis. But in vivo degradation may not parallel that observed in vitro so that the hypothesis may still be valid. The demonstration of partially degraded molecules of an immunogen [Garvey & Campbell (22)] in liver cells, some weeks after injection of the native substance, favors the hypothesis. These degradation products, though they did not exhibit full precipitating activity with the homologous antiserum, inhibited Ab precipitation if they were mixed with the serum prior to the reaction with the complete Ag.

It is probable that during prolonged immunization fresh Ag reacts with the circulating Ab and that the Ag-Ab complex plays a role per se, different from the Ag alone [Najjar et al. (23); Weigle (24)]. It remains to be seen whether the specificity of the complex differs from that of its components.

Nonspecific Factors

It is well known that the physiological state of the recipient controls the immune response. Recently, attention has been drawn to certain substances which enhance this response. It has been shown [Landy & Shear (27)] that certain pyrogenic polysaccharides from bacterial, vegetal, or animal origin can be potent stimuli of Ab formation. These substances, administered 24 hr. before a primary stimulus, lead to the acceleration of Ab production in

such a way that agglutinating Ab can be detected a few hours after injection [McKenna & Stevens (28)]. Obviously, this finding may have an important bearing on immunization procedures. Such nonspecific stimulation of the immune response may also explain the role of certain substances contained in adjuvants as, e.g., the tubercle bacilli used in Freund's adjuvant.

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The fate of the immunogen in the body, i.e., its location, its association with body constituents, etc., may give us a clue to the mechanism of Ab production. To follow this fate, immunogens tagged with radioactive isotopes (externally, with I¹³¹ for example, or internally in the molecule) have been used. It is advisable, though, to combine this method with more specific techniques of individualization, such as immunochemical detection.

After the intravenous injection of a foreign substance, its elimination curve follows three steps: first, a very rapid fall (equilibrium in the fluid space); second, a slow decrease; third, a rapid decline. This third period is usually attributed to the beginning of Ab production at a significant rate and it is during this phase that circulating Ab is first detected, as measured with isotopically labelled proteins [Talmadge et al. (29); Cinader & Dubert (30); Weigle (31, 32)]; or by immunochemical methods [Cinader (33)]. This picture is valid only for heterologous native proteins since, for example, coupling diazocompounds with the protein results in a great increase of its rate of disappearance [Dixon et al. (34)].

The intracellular detection of the immunogen which has given rise to many investigations has recently been reviewed by Campbell (35). For the time being, the results of these efforts seem rather contradictory. It could be that these contradictions arise from the fact that the fate of an immunogen may vary greatly with its nature (whether native protein, chemically modified protein, insoluble particle), the route of injection, and so on.

Although the problem is still unsettled, some interesting observations should be mentioned [see Wissler et al. (36)]. It has been recently found that if a site is injected with immunogen plus Freund's adjuvant and if the region of injection is excised one hour after administration, Ab production still follows [Freund & Lipton (37)]. It must then be concluded that the diffusion of an immunogen throughout the body is very rapid even when it is administered in a localized area.

At the cellular level the immunogen has been detected histologically by means of fluorescent Ab [Coons et al. (38)], through its own radioactivity [Dixon (39); Haurowitz et al. (40); Garvey & Campbell (41); Ingraham (42)] and by taking advantage of its specific immunological reaction with homologous Ab [McMaster & Edwards (43)].

⁴ Substances which associate with the immunogen, in addition to stimulating non-specific reactivity, are used in adjuvants [Ramon (25); Freund (26)]. The potentiation thus obtained emphasizes the importance of the physical state of the immunogen as well as the role of the nonspecific factors in the Ab response.

The immunogen has been found in macrophages and in the cell of the RES soon after injection.

At the subcellular level, it seems that the immunogen has been detected in association with the mitochondria [Fields & Libby (44); Ingraham (45); Erickson *et al.* (46)]. It has even been reported by Garvey & Campbell (47) that BSA-sulfanilate was found associated with RNA from the liver cells.

THE EFFECT OF X-RAY IRRADIATION ON THE IMMUNE RESPONSE

The idea that Ab formation is a multistage process is an old one. It has received increased attention as a result of recent studies on the influence of irradiation on Ab production. It was shown, both for soluble Ag [Dixon et al. (48)] and for red cells [Taliaferro et al. (49)] that a short phase of Ab production, called the "preinduction phase" is radiosensitive [Taliaferro (50)]. After irradiation three to four days must elapse before an animal recovers its ability to respond to an immunogen. Twenty-four hours after irradiation, primary injection of red cells fails to elicit any hemolysin response. The fact that with appropriate timing irradiation damage does not interfere with Ab production suggests that Ab suppression does not depend on a direct destruction of the Ab-forming site.

Attempts have been made to correlate specific radiosensitivity of cells with their role in Ab production. For example, it has been recently shown by Speirs (51) that eosinophils practically disappear from the circulation after whole-body irradiation, but one cannot be sure whether this is a simple correlation or a true causal relationship.

More significant is the finding that Ab production could be maintained by lead shielding of the spleen during irradiation [Jacobson & Robson (52)]; or by administration, after irradiation, of isologous or heterologous spleen cells [Jaroslaw & Taliaferro (53)]; or isologous or heterologous bone marrow cells [Makinodan & Gengozian (54)]. HeLa cells (53) were also effective, as was acellular yeast autolysate (53). The activity of yeast autolysate may be of great practical as well as theoretical importance. It suggests that certain substances may be added with the immunogen in *in vitro* studies to establish artificially the preinduction phase. The activity of yeast autolysate might also be related to the observation [Stender *et al.* (55)] that nonspecific stimulation of the RES one hour after irradiation suppresses the inhibitory effect of this irradiation upon Ab formation.

The two-step theory of Ab formation does not preclude the possibility that all stages of Ab production occur in the same cell or in the same clone of cells. The main experimental approaches to this question have been: (a) as mentioned, to study the cellular pattern in relation to Ab production as, for example, following irradiation; (b) to study the fate of the immunogen or the production of Ab [Coons et al. (56); Leduc et al. (57)]; or γ -globulins [Ortega & Mellors (58)] by the use of immuno-histochemical methods; (c) to study

Ab production employing cell transfer methods [Landsteiner & Chase (59); Harris et al. (60)].

Though it is impossible, as yet, to see clearly the sequence of events, which begins with the introduction of the immunogen into the body and ends with Ab production, I believe many immunologists would agree [see Wissler et al. (36)] on a few points. First, at least three types of cells may play a role in the process: the macrophage, the lymphocyte, and the plasma cell. Second, the Ab-forming cell is a large undifferentiated cell derived from some kind of fixed reticular cell. The fact that the lineage of these different cells is still obscure and that the terminology employed is not standardized, leads to a considerable amount of confusion in this field.

ANTIBODY SYNTHESIS BY CELLS ISOLATED FROM THEIR ORIGINAL MILIEU AFTER IN VIVO SENSITIZATION

The phase of production of Ab can be separated from the phase of sensitization of the cells. This can be done by isolating the cells from their original milieu after they have received, in the normal fashion, the "information" about the Ag structure and have begun to synthesize Ab, either in the primary or secondary phase. In the main, two techniques have been employed for this purpose.

TRANSFER OF CELLS TO FRESH RECIPIENTS

This work was pioneered in 1930 by Topley (61) by the transfer of spleen tissue from an immunized rabbit to a fresh recipient. Later, Landsteiner & Chase (59) demonstrated Ab production by transferring cells from a hypersensitive donor to a fresh recipient. Though the hypersensitivity reaction raises a special problem as it is not associated with detectable amounts of circulating Ab, there is little doubt that it is essentially an immunological reaction. A similar method devised by Rosenberg et al. (62), makes use of the passive cutaneous anaphylaxis reaction. With this method an attempt has also been made to calculate the number of Ab molecules manufactured per cell transferred.

The passive transfer of "transplantation immunity" [Mitchison (63); Billingham et al. (64)] by cells of an actively immunized donor, though it does not provide a favorable approach to quantitative analysis, presents some great advantages in terms of specificity of the reaction to grafts.

The conditions under which transfer of cells is accomplished bear on the following factors in Ab synthesis: (a) the specificity of the reaction; (b) its kinetics; (c) the factors associated (humoral and others); (d) the types of cells involved, etc. [see Tremaine (65) and Waksman & Matoltsy (66)].

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Recent studies concerning circulating Ab to more or less defined immunogens have made use of recipients supposedly nonparticipating in Ab synthesis: "incompetent" recipients. This state has been produced by x-irradiation, immunological nonspecific deficiency (young animals), im-

munological tolerance, and humoral isolation [anterior chamber of the eye: Tremaine (65)].

A group of workers [Harris et al. (19, 60)] have systematically and carefully studied the kinetics, specificity, and quantitative formation of Ab against bacterial Ags by rabbit lymphocytes transferred to an x-rayed recipient. It has been shown that lymph node cells of immunized rabbits synthesize antibovine γ -globulin or antibovine serum albumin Ab, in the recipient rabbit, but that it is not possible to induce, in the x-rayed recipient, or in vitro, a de novo stimulation under these conditions [Roberts & Dixon (67)]. Macrophages as well as lymph node cells may be involved in these transfer reactions (68).

In another approach to the use of incompetent recipients, the formation of Ab against bacterial immunogens by transfer of spleen cells to newborn animals (rabbit, chicken, mouse) has been demonstrated [Sterzl (69); Trnka (70); Haškovă et al. (71)].

Different studies have led to the conclusion that in these cell transfer experiments, a de novo synthesis of Ab occurs directly from amino acids [Green & Anker (72); Taliaferro & Talmage (73); Stavitsky (74)]. From many of these experiments it has been estimated that each transferred cell will manufacture approximately its own weight in specific Ab per day. This is a remarkable rate if one considers that probably not all cells transferred are viable, and that not all viable cells could be specifically engaged in manufacturing the type of Ab being studied.

Opinions are more conflicting about the effect of the recipient "milieu" on the capacity of the transferred cells to produce Ab. One group was unable to observe Ab formation by rabbit lymph node cells which were transferred, in the primary response stage, to neonatal recipient [Dixon & Weigle (75)]. The Czechoslovakian workers were, on the other hand, successful [Sterzl (69); Trnka (70); Haškovă & Mitchinson (71)]. However, the difference in these results could be ascribed to the kind of immunogen used and to the stage of response of the transferred cells.

Emphasis has been placed on the use of intact and viable cells in the transfer of activity. However, a number of studies can be cited in which the authors have claimed positive results with the transfer of an acellular fraction. Transfer of agglutinin formation [Michelazzi (76); Šterzl & Hrubešovă (77)] and of hypersensitivity [Jeter et al. (78); Lawrence (79)] have both been reported. Owing to the fundamental theoretical and practical importance of these results, further work is eagerly awaited.

In vitro Synthesis of Antibodies

The formation of Ab has been followed in vitro in surviving or cultured tissue since the work of Ludke (80) and of Fagraeus (81). The development of agglutinins has usually been followed, because of the sensitivity of the agglutination technique. The incorporation of radioactive amino acids into the Ab has been observed by different workers, thereby demonstrating that

neoformation of Ab probably occurred [(82 to 85); see review by Stavitsky (86)].

The main difficulty stems from the relatively small amounts of Ab produced under these conditions. Many controls should therefore be introduced into this kind of experiment, including the demonstration that specific metabolic inhibitors suppress incorporation of the labelled amino acids, and that passive contamination of the specific precipitate has not occurred.

Attempts have been made recently to detect Ab produced by a single surviving cell in vitro. This was done in order to see whether one cell was able to elaborate more than one type of Ab. The first attempt made use of the method of specific immobilization by homologous Ab of Salmonella suspended in microdroplets [Nossal & Lederberg (87)]. It was found that after an immunization with two strains of Salmonella, no single cell was able to produce the two types of Ab studied. Before this conclusion can be accepted it must be shown, by methods similar to the one used in the microdroplet test itself, that there is no cross reactivity between the Ags employed. These results however are in keeping with the results of histo-immunochemical studies done with two different dyes [Coons (88); White (89)].

Another method employed in the study of Ab production was to measure phase inhibition by Ab synthesized by one lymph node cell suspended in a microdroplet [Cohn & Lennox (90)]. This method, which has the highest possible sensitivity and specificity, has proven in a significant number of cases, that one cell was able to produce two types of Abs directed against immunologically unrelated phages.

ANTIBODY SYNTHESIS BY CELLS SENSITIZED IN VITRO

In the studies cited above all the cells concerned received their specific sensitization in vivo. In vitro sensitization has also been studied with some success and brings the immunologist closer to his ultimate goal. After sensitization of cells in vitro the immunologist has two possible approaches: either to transfer the cells to an incompetent recipient or to try a complete in vitro synthesis.

TRANSFER TO A RECIPIENT

A concerted effort to produce sensitization in vitro has been progressively developed by a group of workers [Harris & Harris (91), Harris et al. (92, 93)]. The relevant features of the system employed by these workers and some of their findings are: (a) The immunogen is a soluble substance of bacterial origin; (b) Preliminary incubation of the bacteria in serum or their trypsination is necessary for in vitro sensitization; (c) The immunogenic fraction is a substance relatively small in size (it is dialysable through cellophane); (d) The sensitizable cells are derived from lymph nodes (rabbit) and are essentially lymphocytes (90 per cent); (e) The recipients are x-rayed and are unable by themselves to manufacture Ab; (f) The yield of Ab produced

(computed from agglutination titer) shows that true neoformation has occurred; (g) This neoformation has been ascribed to the cells transferred, not to a recovery of the recipient's competence as a result of some acellular factor derived from the donor cell. The evidence supporting this latter assertion was obtained by the clever method of preimmunizing an unirradiated recipient with lymphocytes of the future donor. When this animal was challenged with sensitized cells of the same donor, Ab formation was suppressed or greatly decreased [Harris & Harris (94, 95)].

The features of the system described in (b) and (c) may explain native protein inactivity in in vitro sensitization although it is immunogenic in the whole animal. As stated previously, immunogens may be subject to enzymatic degradation [Lapresle & Durieux (20)] at an early stage of Ab synthesis. The fact that the immunogen must be pretreated when lymph node cells are employed suggests that these cells are involved only in the second stage of Ab synthesis in the body. That another cell type(s) is normally involved is suggested by the ability of native immunogen (BGG) to sensitize spleen fragments [Stevens & McKenna (96)] which contain a mixed population of cells. The presence of a protease in the spleen system has an obvious parallel in the pretreatment requirement.

Other studies of sensitization have been made in which, following their contact with bacterial immunogens in vitro, rabbit spleen cells were transferred into newborn rabbits [Sterzl (97); Holub (98)], or hen spleen cells transferred to chicks [Trnka (70)]. In both cases Ab was formed.

In still another system, rabbit lymphocytes were incubated with bacterial immunogens and then transferred to the newborn (98).

SYNTHESIS In Vitro

A complete *in vitro* synthesis of Ab has been recently claimed (96) with the use of a soluble immunogen, BGG, incubated with rabbit spleen fragments. We may advance some hypothesis about the factors which, in conjunction, led to positive results.

The first factor to be discussed is the influence of Salmonella endotoxin. Endotoxin was successfully employed either by administering it to the donor prior to removal of its spleen or by adding it along with the immunogen to spleen fragments in vitro.⁵ A possible mode of action for endotoxin would be the stimulation of protease activity in the spleen. The significance of endotoxin in in vitro Ab synthesis is underlined by the fact that, with one exception [Fastier (99)], bacterial immunogens containing endotoxins were employed in all successful attempts [Przygode (100); Schilf (101); Kimura (102)].

A second factor which appears to influence in vitro synthesis is the source of serum added to the culture medium. Autologous serum appears to be

⁵ Other results from that work suggest that endotoxin exerts its influence *in vivo* on the initial step(s) in Ab synthesis.

superior to isologous serum. This finding seems to reflect a general feature of the system, namely, that the closer one approaches to the *in vivo* milieu, the better are the chances of success.

A comment may be made here about these experiments: while it is true that the titers of hemagglutination are relatively low—too small to apply the *in vitro* amino acid incorporation technique—it is difficult to visualize

any other interpretation than de novo synthesis.

A peculiar feature of these experiments is the fact that, although the rate of Ab production is high during the first few hours, a plateau is rapidly reached. It is possible and even likely that cells enter a nonphysiological state rather rapidly. The use of large fragments (over 100 mg.) probably limits the percentage of cells which can be adequately aerated and which are actually involved in Ab synthesis. In view of this consideration the rate of production per cell is probably much larger than can be estimated from a total cell count. Values for Ab synthesis of 0.01 to 0.1 per cent of the cell weight per day were obtained in other experiments [Rosenberg et al. (62)] in which the measurement appears to reflect the true capacity of the cell. If these values are applied to the *in vitro* system one may calculate that one per cent of the cells in the fragments are synthesizing Ab.

The experiments cited above should be repeated and extended because of their great significance. From the studies already available, however, both on the sensitization process and the subsequent synthesis of Ab, it appears that Ab formation can be fruitfully approached at the cellular level.

THEORIES ON ANTIBODY FORMATION

Although the state of knowledge in immunology is very primitive, many theories on the formation of Ab have been elaborated. Until the present time, it has been very difficult to design experiments with which to test the theories in an unequivocal manner. The theories have been arbitrarily summarized in Table I.

As it is impossible to relate the historical development of the theories on Ab production since the beginning of this century [see Talmadge (103)], we

must limit the discussion to very few topics.

There is a clear relationship between the concept of the unitarian theory of Ab structure and the proposal of the template theory [Breinl (107); Mudd (108); Pauling (109)]. The diversity of possible antigenic structures and strict specificity of the Ag-Ab reaction necessarily lead to an induction theory. Considering the origin of the structural information, the IIa & IIb theories are similar. Their differences arise essentially from the need, in the template theory, for the continued presence of the immunogen itself in the Ab-producing cell. This question of retention, the cause of a great number of discussions, has been especially irritating since it has been impossible to settle this point experimentally. For the time being, one cannot prove or disprove the presence of *one* molecule of immunogen per Ab-producing cell.

TABLE I

THEORIES ON ANTIBODY FORMATION

I—SELECTION	
(a) Complete pre-existence of the information.	∫Ehrlich (104)
Selection at a cellular level.	Burnet (105)
(b) Selection at a molecular level involving suc-	•
sessive steps of increased fitness.	Jerne (106)
II—INDUCTION	
(a) Information is kept in the antigen during	Breinl & Haurowitz (107)
all Ab production.	Mudd (108)
"Template theory."	Pauling (109)
	Campbell (35)
(b) Information is transferred to	Burnet (110)
autocatalytic intermediary units.	Schweet & Owen (111)
III—MIXED HYPOTHESIS	
First step: induction of a specific structure.	∫a/Monod (112)
Second step: selection of the antigen	b/Pappenheimer (113)

Furthermore, the continuous presence of the immunogen is not itself a decisive proof of its necessity for the process of Ab synthesis.

The IIb theories (Burnet, Schweet) imply a self-reproduction mechanism for the information to be transferred to the daughter cells. This reproduction could occur through the intervention of DNA [Schweet & Owen (111)], or RNA [Burnet (110)] intermediaries. These postulates are necessary to overcome the difficulty of considering a protein as a self-duplicating unit, a process unknown in biology today.

Our views about Ab production can be obviously greatly modified if we assume a considerable heterogeneity in the Ab molecule population after immunization with a single immunogen. Three general findings have emerged recently in support of this idea: (a) heterogeneity in a population of antihaptenes Abs [Eisen & Karush (114); Talmadge (115)]; (b) extensive cross-reactivity between taxonomically distantly-related immunogens [Dixon & Maurer (116)]; and (c) the presence of molecules, in the serum of non-immunized animals, possessing a natural affinity for certain structures [Jerne (117)]. These findings have led to the revival of the selection theory.

One must still remark that the above-mentioned concept of heterogeneity in an Ab population still requires proof that this population is composed of molecules specifically directed against one determinant group only. As long as one employs immunogens containing different determinant groups the heterogeneity of the Ab population may reflect the heterogeneity of the immunogen. This particular criticism will always apply to studies in which natural immunogens [e.g., proteins or polysaccharides; see Raynaud (118)]

are used and, in fact, extends to haptenes which usually contain more than one determinant group. With the method of equilibrium dialysis of radioactive haptenes it was possible to use very small concentrations of these haptenes (10⁻⁸) and, thus, to select those Ab sites which have the highest affinity for them [Dubert (119)]. It was found that this type of Ab molecule formed a highly homogeneous population.

However, while the heterogeneity of Abs toward a single determinant group has yet to be proved this concept has fostered a purely selective

theory of Ab formation.

In the selection theory proposed by Jerne (106), the specific globulin molecule brought to an Ab-producing cell is reproduced. This process has no parallel in our knowledge of protein synthesis. It is for this reason that Burnet (105) has recently proposed a clonal selection theory. This theory, related as it obviously is to the type IIIb hypothesis, has a sounder biological basis since it relates the formation of Ab after stimulation to the multiplication of a clone derived from a selected cell. Unfortunately, it loses one of the more tempting features of the Jerne hypothesis: the increased fitness of the Ab molecules through successive selective steps. If it is true that one cell can produce more than one type of Ab this theory will be seriously shaken.

As is usually the case in fields where factual evidence is scarce, the most valid part of these theories (I and II) are their criticisms of the opposing hypotheses.

The introduction of the concept of enzyme induction in biology had a profound influence upon the thinking in immunology because of the general similarity between the two processes. Burnet (110) has considered it in the development of his theory (IIb). However, the differences between the two mechanisms have been clearly underlined by Monod (120). Two especially important characteristics of enzyme induction are, in our opinion, (a) The number and the structures of the different enzymes which can be induced within one species of bacteria is limited; (b) The whole process of induction is under strict genetic control. The first point seems to bring Ab synthesis and enzyme induction into opposition, although it may be that the number of possible configurations which immunogens may have is smaller than was first thought.6 In turn, this would imply that the total number of different determinant groups on the Ab molecules is limited. As for the second point, no genetic control has ever been demonstrated in specific Ab synthesis. If it exists at all it would manifest itself mainly by differences in immunizability between individuals.

A further step in the understanding of enzyme induction came recently from the discovery of the permease [Cohen & Monod (122)]. This factor (also

⁶ It has been speculated, recently, that they could be limited to 50,000 for all protesns! [Haurowitz (121)]. The basis of the calculation, though, is highly hypothetical.

inducible) provides the cell with a mechanism for concentrating the inducer, thus rendering this cell sensitive to much smaller concentration of inducer. This fact, together with the experimental findings of Coons (56) which indicate that, during the secondary response, certain cells seem to fix immunogen more intensely and more rapidly, led some workers to suggest a new hypothesis of Ab formation which I will call the "mixed" hypothesis (Table I). They postulate two steps in the Ab formation. In the IIIa version [Monod (112)], the first step is inductive and implies neoformation of a specific structure on the cell surface, adapted to the antigenic determinant. This "cell bound" factor is distinct from the Ab of classical type. As a result of this first process the induced cells could concentrate the immunogen (select it), this concentration in turn increasing the endocellular synthesis of Ab.

In the IIIb version [Pappenheimer (113)], especially shaped to explain hypersensitivity, a clonal selection hypothesis is added to the previous ideas. The preinduced cell will react to a new amount of immunogen by increased proliferation, thus developing a specialized clone adapted to the synthesis of a certain type of Ab.

The "mixed" hypothesis is compatible with the template theory for the formation of the normal type of Ab. Its principal advantage is to explain the secondary response, which has always been a major stumbling block for the template theory in its usual form. While these conceptions are nothing more than working hypotheses and present many difficulties, they have a rare quality: they can be subjected to experimental check, especially with improved techniques of producing Ab in cell cultures. If it is proven [Cohn (90)] that one cell can produce more than one type of Ab, the specificity of induction will have to be considered at a subcellular level. It will still be possible, though, to visualize, in the light of the IIIb version (Pappenheimer), a mechanism by which any immunogen adapted to an induced cell will trigger its division. This will be followed by the formation of all types of Ab the derived clone is capable of synthesizing, a process which will be recognized as a nonspecific anamnestic response. The Pappenheimer hypothesis also deals with the problem of hypersensitivity. It is visualized that the surface material which presumably concentrates the immunogen is the "cell-bound Ab" which is postulated in the hypersensitive state.

All of the knowledge which is beginning to accumulate on protein synthesis by subcellular fractions can and should be incorporated in any theory of Ab formation [Crick (123)]. These concepts have been applied to the problem of the production of a new protein by Schweet and Owen in theory IIb.

An immunological phenomenon which has not yet been explained by any known theory is that of immune tolerance. Most proponents of the template theory explain immune tolerance by the persistence of the specific substance in the Ab-producing cells which, in turn, traps any Ab molecule produced; the subsequent fate of this Ab has still to be specified. Apart from the fact that this idea is almost impossible to check, one must admit that we still have to explain why the same stimulus leads to Ab formation in the adult and to specific inhibition in the embryo. Though Burnet's first hypothesis has been proposed in great part to explain immune tolerance, it is not easy to see how the "recognizing units" function as inhibitors when induced in the young and as active producing units in the mature animal.

The Jerne hypothesis Ib claims to explain immune tolerance but, again, not satisfactorily. The gamma globulins with an affinity for substances administered to newborn animals will be eliminated. But, why is it that such a structure is not produced again sometime during the life of the animal if we assume, as Jerne does, that random formation of γ -globulin goes on indefinitely? If such a structure was produced, then the whole cycle of Ab formation would start again in the presence of the immunogen. This objection also holds true for any clonal selection theory (Ia).

In short, any theory of Ab production must explain immune tolerance which, in turn, is to say that it should explain a specific discontinuity in the functioning of the Ab-producing system. At one stage, contact with a specific structure "teaches" the future RES, permanently and specifically, not to respond by Ab formation. At another stage, the same contact teaches this system to respond by Ab synthesis. If we do not postulate that the presence of the Ag itself is necessary to maintain this inhibition, then we must postulate some sort of storage of the information to perpetuate the inhibition.

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UNICELLULAR CLOCKS1

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INTRODUCTION

It is well known that various biological processes in both animals and plants are timed with respect to the daily, tidal, monthly, and annual cycles of the environment. In recent years it has become increasingly clear that, at least in many cases, this timing is achieved by a biological clocklike mechanism.

Much of the evidence concerning biological clocks is derived from studies of so-called persistent or endogenous diurnal rhythms. Such rhythms continue to occur, with a period of about 24 hr., even though the light intensity and temperature are kept constant. Rhythms which cease to occur when the organism is kept in constant conditions are known as exogenous rhythms, but most diurnal rhythms appear to fall into the endogenous category, particularly in cases where a careful study has been made of the conditions necessary for the persistence of the rhythm. A diurnal rhythm may take the form of a continuous variation, as in the rate of a particular process, with a maximum at a well-defined time; or a rhythm may be evident from an event which occurs only at one particular time of day. A large part of the extensive literature concerned with biological rhythms and cycles has been covered in review articles (8, 9, 18, 20, 34, 36, 53, 65, 67, 68, 84), and we shall consider here studies of persistent diurnal rhythms in unicellular forms. In this connection, the striking similarities between diurnal rhythms in unicellular forms and those in higher organisms will be discussed.

Persistent rhythms have been described in several unicellular forms. It is interesting that, as in other cellular processes, specialization is found in metazoans. Harker, for example, has shown by transplantation experiments that the clock in cockroaches is located in the suboesophageal ganglion (35), and hormones are known to be involved in clock function in several organisms. In addition, the more spectacular and sophisticated functions of biological clocks are found in higher organisms where an adaptive significance in the clock function is apparent in many cases. In *Drosophila*, for example, the time of emergence of the adult from the pupal case is critical to the organism, since the not-yet-fully-tanned cuticle is quite permeable and extensive loss of water is possible. Emergence is timed by an internal mech-

¹ The survey of the literature pertaining to this review was concluded in December, 1958.

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anism so that it occurs just before dawn, when the daily temperature is lowest and humidity is highest (52, 66). Bees possess a true sense of time which enables them to return at a precise time of day to a feeding station, discovered at that time on the previous day (72, 83). In birds and arthropods, there is evidence that celestial navigation is achieved by continuous compensation for the changing position of the sun in the sky, indicating that the animals possess a clocklike mechanism (55, 62, 63, 64). Bünning (17, 18, 19) has reviewed evidence that a clock-mechanism may be the basis for various photoperiodic phenomena in higher plants.

Despite the metazoan elaborations in localization and function, it is clear that the rhythmic mechanisms in diverse kinds of organisms have similar properties, insofar as the clocklike characteristics are concerned. Thus, while the adaptive significance of rhythms in unicellular forms may not be so evident to us, the properties of the rhythms demonstrate the existence of a basic clock-mechanism which may be ubiquitous among organisms.

PERSISTENT ENDOGENOUS RHYTHMS IN UNICELLULAR ORGANISMS

Although they provide favorable material for study at the subcellular and chemical level, unicellular organisms have been used relatively little. Probably the first unequivocal demonstration of a persistent diurnal rhythm in a unicellular form was that of Pohl (70), in 1948. He observed a daily rhythm in the phototaxis of Euglena gracilis, the response being maximal in the middle of the day and minimal at night. The studies of Bruce & Pittendrigh (12, 13, 14) have shown that this rhythm persists for weeks with a period which is close to 24 hr.; that the period is essentially temperature-independent; that the rhythm may be entrained to light-dark cycles; and that the phase may be changed by a single exposure to light. These authors have directed attention to the fact that the cellular level of organization is sufficient for a biological clock with these essential clocklike features.

Paramecium exhibits a diurnal rhythm of sexual activity (22, 48, 74). The time of day at which the mating reaction is strongest varies in different species and varieties. The rhythm in variety 1 of *P. bursaria*, where the mating reactivity becomes maximal at the middle of the light period, has been extensively studied by Ehret (22 to 25) and shown to persist for as long as one week in continuous darkness. Other clocklike features of this rhythm will be discussed in a later section. It is also possible that cell division in *Paramecium* shows a diurnal rhythm, but this has not been studied extensively (51).

An extremely fascinating rhythm in *Paramecium multimicronucleatum* has been described recently by Sonneborn & Sonneborn (75), involving an actual change in the mating type of the organism. When stock 232-6 is exposed to alternating periods of 6 hr. of light and 18 hr. of darkness, it is mating type IV during the last 3 hr. of light and the first 12 hr. of darkness, but mating type III during the first 2 hr. of light. During the interim periods, variations in response have been observed. Several characteristic features of persistent rhythms were reported. The rhythm persists for at least three days in continuous darkness; the same cycle is observed regardless of the

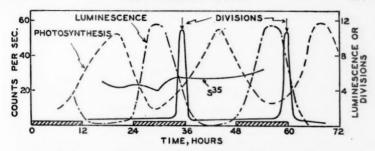


FIG. 1. This figure illustrates diurnal rhythms of luminescence, photosynthesis and cell division in cultures of *G. polyedra*, kept under conditions of alternating light and dark periods of 12 hr. each at 25°C. The dark periods are indicated on the abscissa by shaded rectangles. Ordinates for luminescence and cell division are in relative units. Each of the rhythms shown persists when the cells are transferred to constant conditions, as shown in Figure 2 for the rhythm of luminescence. Rhythms of a spontaneous luminescent flashing and of a steady glow have also been observed (44) but are not shown here. The curve labeled S³5 illustrates an experiment in which it was found that the rate of inorganic sulfate incorporation does not show a diurnal rhythm.

relationship between solar time and the time schedule of the artificial lights; and a phase shift of the rhythm occurs following an appropriate exposure to light, brighter light being more effective than dimmer.

Studies in our laboratories with the marine dinoflagellate, Gonyaulax polyedra, have shown that it possesses persistent diurnal rhythms of bioluminescence, of cell division, and of photosynthesis, each of which continues with a period of approximately 24 hr. when the temperature and light intensity are held constant (39, 44). Of additional interest in this case is the fact that the phases of the different rhythms are different, i.e., the maximum of each occurs at a different time of day, and that these phase differences are retained in cells maintained under constant conditions. Figure 1 illustrates these rhythms and the phase relationships involved, and Figure 2 shows the persistence of the luminescence rhythm in cells maintained under constant conditions.

There are a number of reports in the literature concerning such diurnal rhythms in marine dinoflagellates. A persistent daily rhythm in the "phosphorescence" of the ocean was observed by a number of investigators (37, 54, 59, 86), and the early studies are reviewed in Harvey's monograph (37). Quantitative measurements in our laboratories have demonstrated a persistent rhythm of luminescence in cultures of G. polyedra (43, 46, 47, 79), in which the light emission may be from 60 to 100 times greater at night than by day. Gonyaulax monilata also exhibits a persistent diurnal rhythm of luminescence (78) but, on the other hand, the colorless phagotrophic marine dinoflagellate Noctiluca miliaris does not (38, 45, 60).

Rhythms of cell division have been reported in several marine dinoflagel-

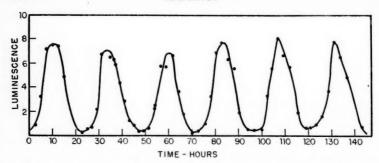


Fig. 2. The rhythm of luminescence in *G. polyedra* from cultures maintained in constant dim light and constant temperature (120 ft. c., 21°C.) The average period was 24.4 hr. and, under these conditions, no apparent damping occurs. If the cells are, instead, left in the dark, as in Figure 3, there is a progressive amplitude decrease, but the natural period is the same.

lates. Gough (33) and Jörgensen (50) observed that members of the dinoflagellate genus Ceratium from plankton samples divided only at night, the maximum number of divisions occurring at about 3:30 a.m. in C. fusus, and between 3:00 and 7:00 a.m., in C. tripos. In cultures of Peridinium triquetrum, Braarud & Pappas (4) found that a maximum in cell division occurred between 9 and 10 a.m. Whether or not these rhythms were of a persistent nature was not determined, but a similar cell division rhythm in Gonyaulax has been found to persist under constant conditions (80). In cultures maintained in alternating light and dark periods of 12 hr. each, the maximum in cell division occurs just at the end of the dark period, essentially at dawn (Fig. 1). More recently, a similar rhythm in Gymnodinium splendens has been observed (78).

Evidence for a daily rhythm in the rate of photosynthesis in phytoplankton samples taken from the sea was obtained by Doty & Oguri (21) and Shimada (73). The rate of light-dependent C¹⁴O₂ incorporation in uniform samples incubated under identical conditions was about six times greater in the early morning than in the evening. Measurements showing appreciable daily variations in the chlorophyll content of natural phytoplankton populations (73, 85) suggested that the rhythm might result from chlorophyll variations. Although no mention was made of the kinds of photosynthetic organisms present in these samples, one would presume that various species of dinoflagellates and other unicellular forms were represented. A similar rhythm of photosynthesis has recently been observed in our laboratories with cultures of Gonyaulax (39, 40). In cultures grown in alternating light and dark periods of 12 hr. each, the maximum rate of C¹⁴O₂ incorporation fell at the eighth hour of the light period (Fig. 1). The rhythm is a persistent one, since the variations continue when the cells are kept under constant condi-

tions. In contrast to the studies mentioned above (73, 85), no concomitant diurnal variations in either chlorophyll or carotenoid content were found.

A diurnal rhythm in the rate of bacterial multiplication was reported by Ogata et al. (61). They observed an acceleration in the growth rate of E. coli between 8 P.M. and 5 A.M. Additional studies will be required to establish the nature of this rhythm.

Fauré-Fremiet has reported that *Strombidium*, *Chromulina*, and *Hautzschia* show a tidal rhythm in their movements, which continue when the organisms are kept in the absence of tides (27, 28, 29). Bracher has reported a similar tidal rhythm in *Euglena limnosa* (5).

CLOCKS, OSCILLATORS, AND BIOLOGICAL RHYTHMS; THEIR FEATURES COMPARED

Several authors, including Pittendrigh & Bruce (67, 68), Brown (7, 9), and Bünning (19), have considered the time-measurement aspect of rhythms, and Pittendrigh & Bruce have developed analogies between biological clocks and oscillators. We will consider here a number of the analogies and comparisons between features of such familiar devices as clocks, oscillators, and pendulums on the one hand and clocks in unicellular organisms on the other hand. These considerations are of value, first, in establishing that an endogenous cellular oscillator which keeps reasonably good time is involved and, secondly, in directing us towards an understanding of the physical and chemical nature of this mechanism.

Natural period.—A familiar oscillating device such as a pendulum or an electronic oscillator possesses a "natural period" which is a characteristic of the configuration of the system. When a given oscillator is coupled to a second oscillator the frequency of which is slightly different, it may be driven so that it is forced to the frequency of the second oscillator (see below). But when this coupling is removed, the first oscillator always reverts to its natural frequency or period.

Biological clocks, which in nature are "driven" by the light-dark changes of the solar day, also possess innate or "natural" periods. It has been found in practically every case examined (43, 67) that, under constant conditions of light and temperature, the period of a biological rhythm differs from the 24-hr. period of the solar day. Depending upon conditions (see below), the natural period in *Gonyaulax* varies from about 23 to 27 hr. [Fig. 2 (42, 44)]. In Euglena, Bruce & Pittendrigh (12) have shown that the period of the rhythm of phototaxis varies from 23 to 26 hr.; and in *Paramecium*, Ehret (25) has found that the natural period is approximately 23 hr. at 25°C.

The period of a diurnal rhythm is an innate characteristic which need not be "learned" by or "impressed" upon the organism. In certain cases organisms which are kept in darkness or constant illumination fail to show a rhythm. For example, the eclosion of *Drosophila* occurs arrhythmically if the the eggs are laid and development occurs in complete darkness. However, if pupae from eggs laid and kept in the dark are exposed to a brief flash of

light (1 min. will suffice), eclosion on subsequent days occurs only at one time of day, the time depending upon when the organisms have been exposed to the flash of light (6, 66, 67). In Gonyaulax, although the rhythms persist in constant dim light, the effect of constant bright light is to cause arrhythmicity (47, 79). The rhythms in Euglena and Paramecium are similarly lost when the cells are kept in constant light (13). If arrhythmic Gonyaulax cells which have been kept in bright light are simply transferred to darkness, a diurnal rhythm is exhibited, and the phase is related to the time when the cells were darkened (79). The characteristic natural period is always observed, however, even though the cells may not have been exposed to diurnal light or temperature cycles for as long as three years (43). These experiments also illustrate another clocklike feature of rhythms, i.e., that setting requires only one change in light intensity, serving to give the phase information.

Further evidence that the period is innate rather than learned is derived from the failure of attempts to change the natural period. By exposing an organism to light-dark cycles with atypical periods, it is possible to "entrain" or "drive" a rhythm so that a maximum occurs once every driving cycle. With Gonyaulax it is possible to obtain maxima in cell division and luminescence every 16 hr. by exposing the organisms to alternating light and dark periods of 8 hr. each. But when the cells are placed in constant conditions, the rhythm reverts to its natural period (43, 44). An experiment of this sort has been carried out in which the cells were grown with a 16-hr. light-dark cycle for seven months. Even after so long a time—between 70 and 100 cell divisions had occurred—the rhythm reverted to its natural period when the cells were placed in constant conditions.

Observations concerning the natural period demonstrate that a mechanism endogenous to the organism is responsible for the timing ability. There are a number of environmental physical factors, such as barometric pressure or cosmic-ray intensity, which may fluctuate with a 24-hr. frequency under the usual "constant conditions" of the laboratory. If the clock system were coupled to these factors in a way similar to the way it can be coupled to light-dark changes, then the frequency of the biological rhythm should correspond to that of these external variables. Since the periods of persistent rhythms are characteristically different from 24 hr., one concludes that such variables are not affecting the frequency of the clock system. Brown, although agreeing that there is an endogenous component in a biological clock-system, argues that the maintenance of a regular frequency is achieved by virtue of some kind of as yet undefined "external pacemaking signal" (8, 9). A principal argument for this view evidently rests on the view that "temperature-independence of a biological phenomenon . . . would pose a formidable problem indeed for the general physiologist" [(8), p. 131].

The temperature-independence of biological clocks is certainly one of their most striking and important features. Pittendrigh & Bruce (68) have cited some 17 organisms in which rhythms have now been shown to be temperature-independent, and the fact that only three of these cases were reported prior to 1954 attests to the recent explicit concern with this functionally important aspect.

Concerning accuracy and inaccuracy: temperature-independence.-Accuracy is clearly an important aspect of a functionally useful clock, which must operate satisfactorily under a variety of diverse environmental conditions. The component parts of a mechanical clock are not temperature-independent, and appropriate temperature compensations are therefore built into the clock. From an evolutionary point of view we would certainly not expect to find a biological clock-mechanism which was accurate at one temperature but grossly inaccurate at another. Moreover, the fact that the rates of single chemical reactions are always affected by temperature does not mean that a chemical mechanism for temperature-independence is impossible. Biochemical feedback systems, in which the rates of specific chemical steps are controlled either through control of enzyme synthesis or enzyme activity, have been described (31, 32, 82). It is also possible, as suggested previously (42), that a mechanism involving two enzymatic reactions, in which a product of the first was an inhibitor of the second reaction, could result in apparent temperature-independence for the second reaction. There are certainly other possibilities which, in the absence of relevant chemical information, need not be considered at the present time.

The temperature-independence of biological clocks refers to the lack of large differences in the period of a rhythm when the organism is maintained under constant conditions at different temperatures. The precise measurement of the period requires a relatively good assay method and a reasonably long-term experiment. In Euglena, Bruce & Pittendrigh found that the period varied from about 26 hr. at 16.7°C. to slightly greater than 23 hr. at 33°C. (12), and they suggested that apparent temperature-independence was achieved by means of a temperature-compensating mechanism within the organism. In our studies with the rhythms in Gonyaulax, the period was found to be longer at higher temperatures, resulting in an apparent Q10 of less than 1.0 (42, 44). At 16°C. the period was found to be 22.8 hr., while at 26.7°C. it was 26.5 hr., and at intermediate temperatures intermediate values were obtained. These results suggested that overcompensation was occurring, and since any temperature compensation mechanism carries the possibility of imprecision, cases of overcompensation are not in a separate class from cases of undercompensation. Another example of a Q10 of slightly less than 1.0 was reported by Bühnemann for the rhythm of sporulation in Oedogonium (16). In Paramecium, Ehret has also found that the period is not greatly different at temperatures ranging from 16°C. to 30°C. but the assay is not well suited to the detection of relatively small differences (26).

It should be emphasized that rhythms are not unaffected by temperature; only the period is independent of temperature. At different temperatures, there are marked differences in the amplitudes of the rhythms in Euglena (12), Gonyaulax (42), and Paramecium (26). Moreover, repeated and nonrepeated temperature changes have been shown to affect persistent

rhythms in a number of ways. Pittendrigh showed that a temperature change in *Drosophila* resulted in transient changes in the period (66), and Ehret (24) and Stephens (76, 77) have demonstrated that a phase shift may be brought about by temperature changes.

Another environmental variable which we have studied in *Gonyaulax* is light intensity. The natural period has been found to differ only slightly at different but constant light intensities (43). The period varied from 22 hr. at 680 ft. c. to 24.5 hr. at 120 ft. c. and in the dark, all at 21°C. These results are similar to those reported previously for the rat and mouse, in which it was found that the natural period was slightly but measurably different at different light intensities (1, 11, 49).

By way of a summary to these comments we can generalize as follows: biological clocks, like familiar physical oscillators, possess a natural period which closely matches the 24-hr. period of the solar day, and the extent to which the period of a rhythm differs from 24 hr. is a measure of the inaccuracy. Biological clocks, like man-make timepieces, are relatively accurate (71), but always inaccurate, under both constant and varying environmental conditions.

On setting and resetting clocks.—A timepiece has a resetting device to correct for inaccuracy or to permit the owner to set his clock to a different longitude. Biological clocks are always found to be reset when corrections of this sort are indicated, so long as the organisms are exposed to the light-dark changes of the environment. The diurnal periodicity in illumination constitutes the principal environmental factor to which the clock mechanism may be coupled although, as mentioned before, it is known that diurnal temperature cycles can act similarly (68).

The rhythm of an organism maintained on a 24-hr. light-dark cycle always shows a 24-hr. period rather than its natural period, so that inaccuracy is corrected for each day. A displacement in longitude may be simulated in the laboratory by scheduling artificial lights to correspond to any desired position on the earth. The phase of the rhythm being studied may thus be caused to bear any desired relationship to local solar day and night. When the organisms are subsequently placed in constant conditions, the rhythm persists with its characteristic natural period, the phase being related only to the time of the previous light-dark schedule. The fact that the phase of the rhythm need bear no particular relationship to local solar time is also evident from experiments in which organisms having rhythms with natural periods different from 24 hr. are left under constant conditions for an appreciable length of time, so that a phase drift occurs.

The converse type of experiment, that of changing the longitude of an organism in the absence of temperature or light cycles, has been carried out in a few cases. Brown et al. (10) transported Uca to Berkeley, California, in continuous darkness and, over the course of six days, found no phase modifications in the rhythm as compared to controls left in Woods Hole,

Massachusetts. Renner (72) did a comparable experiment with bees moved from Paris to New York and obtained similar results.

It is thus apparent that the phase of biological rhythms may be fixed or modified by coupling to diurnal light cycles; but when light and temperature are held constant, no other periodic environmental variables modify phase. Experiments demonstrating these properties have been carried out with a number of unicellular forms (12, 14, 22, 43).

However, recent experiments have demonstrated that, in order to shift the phase of a rhythm, it is not necessary to subject the organism to the corresponding light-dark cycle. Rather, an appropriate single step-type or pulse-type change in light intensity is sufficient. This is comparable to the fact that one only has to set his watch once when he goes from New York to California. Referring to the oscillator analogy developed by Pittendrigh & Bruce (68), it is well known that a single disturbance or perturbation applied to an oscillator quite generally shifts its phase. Thus, a pendulum disturbed once possesses a new phase.

A change in light intensity constitutes the signal by which a biological clock may be reset; or, stated in another way, light is the kind of pertubation which disturbs the cellular oscillator. For example, single $2\frac{1}{2}$ -hr. light perturbations can be so arranged as to shift the maximum of luminescence in Gonyaulax so that it will occur at any desired time of day. Phase shifts following 6-hr. perturbations in Gonyaulax are illustrated in Figure 3.

The ultimate phase of the luminescence rhythm which is attained following a light perturbation is determined by the combination of at least three factors: the intensity of the light perturbation, the duration of the light perturbation, and the time during the old cycle when the light perturbation is administered (43, 44). Higher intensities and longer durations usually result in a more pronounced phase shift, until maximal values are reached. The nature of these relationships is not simple. For example, we have found that a light intensity of about 800 ft. c. is required to obtain the maximum phase shift with a $2\frac{1}{2}$ -hr. perturbation. Yet if the duration of the perturbation is increased to 6 hr., a light intensity of 30 ft. c. is sufficient to give the maximum phase shift (45). The phase shift obtained following a single exposure to a light intensity of about 1000 ft. c. has been found to be proportional to its duration, up to a maximum which was obtained with an exposure lasting about $2\frac{1}{2}$ hr.

The effect of a light perturbation in bringing about a phase shift also depends upon when in the cycle the perturbation is administered. If a light perturbation ($2\frac{1}{2}$ hr., 1000 ft. c.) is given to cells (otherwise kept under constant conditions) during the time of the cycle corresponding to normal day, little or no phase shift occurs; but a similar perturbation during what would be considered the night phase results in a marked phase shift. As illustrated in Figure 3, the phase shift may occur in either of two ways: the subsequent maximum may occur earlier than usual, i.e., one period of less than 24 hr.

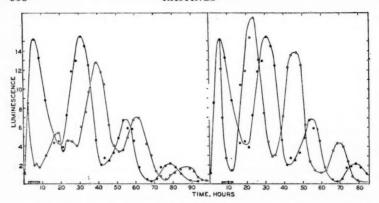


Fig. 3. These experiments illustrate the way in which the phase of the luminescent rhythm is shifted following an exposure of the cells to light (1400 ft. c., 21.2°C.) The control curve, shown by black dots, is the same in both graphs and was determined from cells which were kept in the dark all the time. The other cells were treated identically, with the exception of a 6-hr. light perturbation, given at a time indicated by a rectangle on the abscissa. Note that for the curve on the left (white dots) the light exposure was begun prior to the maximum in luminescence (corresponding to early "night phase"; compare Fig. 1), and that the next maximum was shifted to the right, giving a phase "delay." The curve on the right shows the result when the perturbation was begun about 3½ hr. later, just at the time of maximum luminescence. The subsequent maximum was moved to the left, giving a phase "advance."

duration is observed and the phase is advanced; or the subsequent maximum occurs later than usual so that there is one period longer than usual and the phase is delayed.

It has been found that the phase is delayed if the perturbation is begun at a time corresponding to the late day or early night phase (Fig. 3), the phase shift becoming maximal at a time corresponding roughly to the middle of the night phase. This maximum value approaches 12 hr., when conditions otherwise are optimal. Conversely, light perturbations given at later times in the night phase result in phase shift in the opposite direction, i.e., a phase advance, and the amount of phase shift obtained decreases progressively from a maximum during the middle of the night phase to a minimum during the day.

Single light perturbations of this sort have been shown to reset the clock in every organism studied, including *Paramecium* (23, 24, 25), *Euglena* (14), and *Gonyaulax* (43). It is striking that in each of these cases a phase advance or a phase delay occurs under precisely the same circumstances as described for *Gonyaulax*. This generalization may also be applied to higher organisms, according to Bruce & Pittendrigh (14). These findings suggest that the clockmechanisms in diverse forms of animals and plants are similar. Moreover,

since the phase ultimately attained is not uniquely determined by the time of perturbation, one is obliged to conclude that the clock-mechanism involves a complexity greater than an oscillation in the concentration of a single compound.

Ehret has studied other aspects of the resetting phenomenon in *Paramecium* and has shown that a phase shift is obtained subsequent to short times of irradiation by ultraviolet light (25). Ultraviolet-induced phase shifts may be reversed by subsequent exposure of the cells to white light.

On entrainment.—As mentioned previously, an oscillator may be entrained by a second oscillator if the frequencies of the two are not too different and if there exists appropriate coupling between the two. The phenomenon may involve a mutual entrainment such that the frequencies of both oscillators are somewhat changed from their natural frequencies. The results obtained in a number of experiments concerned with the effect of varying light-dark cycles upon biological rhythms appear somewhat analogous to the entrainment phenomenon of oscillators. For example, the activity rhythm of the mouse may be entrained to light-dark cycles having periods ranging from about 22 to 24 hr., but not beyond (81). Phenomena of this sort have been discussed in some detail by Pittendrigh & Bruce (68) in connection with their oscillator model.

On the other hand, it has been reported for some organisms, including Euglena (13) and Gonyaulax (43, 44), that entrainment of rhythms is possible over relatively wide frequency ranges. In Gonyaulax, luminescence and cell division have been entrained so that a maximum occurred every 12 hr., every 14 hr., or every 16 hr., corresponding to light-dark cycles of 12, 14, and 16 hr., respectively. If two oscillators are rigidly coupled, the first can drive the other no matter what the difference in their natural frequencies, so long as the driving oscillator possesses sufficient energy to do so. The clock-systems of organisms are certainly not so rigidly coupled to the light-dark cycles as to allow any frequency whatsoever to drive, but we have found that the intensity of the light is an important factor in entrainment to cycles which differ considerably in period from 24 hr. In experiments with Gonyaulax where the cells were exposed to 12-hr. cycles (6 hr. light; 6 hr. dark), it was found that entrainment occurred when the light intensity used was 800 ft.c. but that entrainment did not occur when the intensity was 200 ft.c.

On readability.—One must be able to read a clock, but the information is not necessarily of the simple yes or no type. Considering all organisms, no particular phase relationship for diurnal rhythms is either dictated or excluded. One may cite examples of rhythms with maxima at almost any time of day, and the clock of a given organism is such that rhythms of different processes may be phased differently. Luminescence, cell division, and photosynthesis are clocked so that they occur maximally at different times of day in Gonyaulax (39, 44); and in mammals, Halberg and collaborators (34) have found phase differences in different processes. Moreover, a particular kind of rhythmic process need not be phased at a particular time of day. In dif-

ferent species of dinoflagellates, a maximum in cell division has been reported to occur at times ranging from 3 a.m. to 10 a.m. (80); and in different species and varieties of *Paramecium*, the time of maximum sexual activity varies (22). We do not wish to make the generalization too broad, since we would scarcely expect to find an organism where luminescence was maximum at noon and photosynthetic rate maximum at midnight.

The point, then, is that biological clocks possess and confer intelligible information concerning an absolute time measurement. The integration between the clock-mechanism and any particular rhythmic process is evidently such as to allow for a yes or no answer with respect to a given situation. These observations would suggest that the physiocochemical variation in the clock itself involves not only quantitative changes over the course of the day but qualitative changes as well.

CONCERNING THE CHEMICAL NATURE OF THE CLOCK-MECHANISM

There is little information at the present time concerning the physicochemical nature of rhythmic mechanisms; and specific knowledge concerning chemical processes within rhythmic cells is, therefore, of basic value. As a matter of fact, certain kinds of biochemical information could appear distressingly useless in attempting to evaluate possible mechanisms, since at the outset one might have little or no basis for distinguishing a biochemical system which is the clock from a biochemical system which is controlled by the clock. But such a dilemma does not constitute an impasse to our ultimate understanding of the mechanism, since several ways to make distinctions are possible.

As a beginning we need to know just which biochemical aspects of the rhythmic cell show rhythmic fluctuations and which do not and, equally important, what phase relationships are involved. In Gonyaulax, lucifering and luciferase (the substrate and enzyme involved in the luminescent reaction) are found in greater amounts in cell-free extracts prepared during the dark period (41), showing that the rhythm of luminescence reflects rhythmic biochemical processes. Other experiments, however, allow us to ask whether the luminescent system is the clock or is controlled by the clock, and the latter possibility is the most probable (43, 44). These results, therefore, give us some knowledge concerning this part of the system. In examining other biochemical aspects of the Gonyaulax system, we have noted that inorganic sulfate utilization is arrhythmic (Fig. 1), while the rate of light-dependent CO2 incorporation is rhythmic (37). In other preliminary studies, we have found no large differences in the rate of incorporation of phosphorus into DNA and RNA, or indeed into the individual nucleotides thereof. Variations in a small part of the total nucleic acids could not have been detected in these experiments, however.

It is possible that experiments with inhibitors will give information concerning the specific chemical nature of the clock-system. In previous studies, relatively few compounds have been tested (2, 3, 15, 18), and in most ex-

periments the organisms have remained in the presence of the inhibitor [except Ball & Dyke (2)]. For a number of reasons, it is to be expected that inhibitors might have a demonstrable effect through a phase shift, and this only if they are given for a short while and then withdrawn, in a way similar to a single light perturbation. Inhibitors in general have been found to have the effect of damping the rhythm, which, of course, is quite similar to the effect of continuous bright light. It might be possible to mimic the effect of light, with regard to phase shifting, by exposing the cells to a pulse of a specific compound. Studies of this sort should allow us, for example, to identify systems which are controlled by the clock but are not themselves the controlling mechanism.

Pittendrigh, Bruce & Kaus (69) have presented evidence to indicate that a biological clock may be analogous or similar to a coupled-oscillator system. On the basis of studies of the nature of transients which occur following single phase-shifting perturbations to rhythmic systems, they propose that two distinct oscillating systems underlie a particular biological rhythm.

The fact that undamped chemical oscillations may occur in certain systems maintained under constant conditions was shown by Lotka (56, 57, 58). Biochemical and genetic knowledge is now sufficiently sophisticated to provide us with specific examples of how such oscillating chemical systems might occur. For example, Galston & Dalberg (30) recognized that an inducible enzyme system could show rhythmic properties under certain conditions. In the simplest model, an enzyme is induced in the presence of a substrate which itself is destroyed by the induced enzyme. The substrate is produced at a constant rate, and its concentration increases with time. With the formation of increasing amounts of enzyme, however, the substrate concentration is reduced to a low level, so that the rate of enzyme formation is again decreased. Should the enzyme already present be inactivated at an appropriate rate, the system would return to its initial state and the cycle could resume again, with the period determined only by the values of the appropriate velocity constants. Although the enzyme system with which Galston & Dalberg (30) were concerned is in fact apparently noninducible, the general mechanism embraced by the model is applicable to a variety of specific biological processes.

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RECOMBINATION IN BACTERIOPHAGE¹

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Bacteriophages are viruses specific for certain bacteria. The range of bacteria serving as hosts and the morphology of the plaques, i.e., the virus colonies in the bacterial layer on an agar-plate, are features characteristic for each strain of phage. Both qualities are affected by mutational changes, which may arise spontaneously (1, 2) or which may be induced (3, 4, 5). In all well-investigated phages a diversity of plaque-type mutants is available.

Upon a mixed infection of bacterial cells by particles of two or more genetically different types of the same or related phage strains, all infecting types multiply inside the cell and, in addition, give rise to recombinants, i.e., particles carrying markers from different parental types. In 1946 after Delbrück & Bailey had obtained evidence suggesting the occurrence of this genetic recombination (6), the first aim of phage genetics consisted of the collection of detailed data and their understanding in terms of a quantitative analysis. The classical investigations of Hershey & Rotman (7, 8) in T2 were followed by parallel studies in T4 by Doermann and his co-workers (9, 10, 11), in λ by Jacob & Wollman (12, 13) and Kaiser (14), in T1 by Bresch and his colleagues (15 to 18), and in several other phages (19 to 24). From the frequencies of recombinants in 2- and 3-factor crosses, the existence of linkage and a linear order of markers was established in all well-investigated phages.

This first period of phage genetics culminated in 1953 in the formulation of the "mating theory" by Visconti & Delbrück (25), treating a cross experiment in phage as a phenomenon of population genetics. Recently, based on their analysis, a generalized picture was developed, leading to a reconsideration of the population genetics involved. The actual condition of this inquiry will be the main consideration of the review presented here.

Beside this approach through formal genetics, a second line of investigation soon arose with the ultimate aim of relating the genetic phenomena of replication and recombination to features of the molecular structures involved. This attempt was initiated on one hand from two fundamental findings of Hershey & Chase: first, the radio-isotope study of the fate of phage material in the course of infection, which showed essentially that DNA entered the cell (26) and, second, the discovery of partial heterozygotes (27), i.e., particles carrying in a short section of their genome two different

¹ The survey of the literature pertaining to this review was concluded in December, 1958.

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genetic informations from two parent particles. On the other hand, the inquiry was based on the model of DNA structure and replication proposed at about the same time by Watson & Crick (28, 29). Since this beginning many important contributions were added to the problem of recombination in terms of physico-chemical properties of molecules involved, being either experimental observations (30 to 39), or theoretical considerations (40, 41, 42, 44). Needless to say, in spite of considerable progress we are still far from a complete understanding of "molecular genetics" or "genetic activity of macromolecules." For discussions of the problems concerning this line of research the reader is referred to the excellent reviews recently published by Delbrück & Stent (43), Stent (44), and by Levinthal (45). As an important part of this development the investigations of Benzer should be mentioned separately, findings which fundamentally improved our knowledge of the gene structure and of the mechanism of mutation (46, 47). All of these questions, however, cannot be discussed here nor can the problems of radiation genetics of phage, which were excellently summarized by Stahl in a recent review (48).

THE BACKGROUND OF THE POPULATION PROBLEM

In spite of the above mentioned parallelism between the genetic system of phage and higher organisms (linkage, linear arrangement of markers), it became immediately obvious that the recombination process in phage is somewhat more complex. This can be seen from the following experimental facts:

The existence of triparental recombinants.—A bacterial cell can adsorb many phage particles. Therefore, an infection is feasible with more than two genetic types of particles, each of which can participate in replication and recombination. In a "triparental" cross, for instance, the cells will be infected by the phage types ab^+c^+ , a^+bc^+ , and a^+b^+c . In this case one finds triparental recombinants abc among the progeny, i.e., particles with a marker from each of the three parental types [T2: Hershey & Rotman (7); T4: Edgar (37); T1: Bresch (15); λ : Kaiser (14)].

The increasing percentage of recombinants with increasing time of lysis.—In the "standard" phage cross, infected bacteria are diluted and allowed to liberate phage by spontaneous lysis, which occurs at a time characteristic of the phage strain (latent period) (49). It is possible, however, to break up the cells before spontaneous lysis [premature lysis, Doermann (50)] or, in some special cases, to prevent lysis from occurring until considerably after the normal latent period [lysis inhibition, Doermann (51)]. With premature lysis in the first half of the latent period, no infectious (mature) particles are found. Later. they appear in an approximately linearly increasing number which reaches, with lysis inhibition, about double the value of normal lysis. In all cases it is found that among the liberated mature progeny the fraction of recombinants steadily increases the later lysis occurs. For example, a particular pair of markers will show recombination percentages of

about 1.5 per cent, 3 per cent, and 12 per cent for very early, normal, and delayed lysis, respectively [T4: Doermann (9); T2: Levinthal & Visconti (52); T1: Bresch & Trautner (17)].

Negative interference among the progeny of a cross.—We focus our attention on the class of phages which are recombinant in a given region; the probability of these phages to be also recombinant in an adjacent region is found significantly greater than in the entire population. In other words, recombinational events are not distributed at random among the progeny of a cross but, rather, these events appear correlated [T2: Visconti & Delbrück (25); T4: Doermann (9), and Chase & Doermann (36); T1: Bresch (34); λ · Jacob & Wollman (12)]. This "negative interference" is likewise dependent on the time of lysis, showing a greater "factor of coincidence" in early lysis [T1: Trautner (53)].

Ratio of minority recombinants to minority parents.—The infection of cells can be symmetrical by a statistically equal amount of all infecting types, or asymmetrical by an unequal average number of different infecting types. In the case of asymmetric infection with two types, we speak of a majority and a minority parent (25). In such asymmetric 3-factor cross one can also distinguish between "minority and majority recombinants," where minority recombinants carry two markers of the minority and one marker of the majority parent, and vice versa. With strong asymmetry more minority recombinants than minority parents are found among the progeny after normal lysis [T4: Doermann & Hill (11); T2: Visconti & Delbrück (25)].

These facts led Visconti & Delbrück to treat a phage cross as a phenomenon of population genetics (25). From the sum of experimental evidence given here and from a number of biochemical and radiological experiments as well, one can today draw the following picture of the life cycle of a virulent phage, which is essentially the model suggested by Visconti & Delbrück (25): the parental phages adsorb to the host cell, and their genetic determinants [very probably DNA, Hershey & Chase (26)] enter the cell where they first form noninfectious entities [Doermann (50)] known as "vegetative" phage. In addition to performing necessary physiological functions, such vegetative phages are also able to reproduce their genetic information geometrically [Luria (30] and to interact with each other, thus giving rise to genetically recombined structures. When the so-called "pool" of the vegetative particles reaches a certain size, some of the particles begin to mature into infective phage which do not participate in any further reproductive or recombining processes within the cell. The rate of maturation matches at least approximately the rate of reproduction, so that the pool of vegetative phage remains more or less constant. The processes of reproduction, recombination, and maturation continue concomitantly until terminated by lysis of the cell.

³ The factor of coincidence is the quantitative measure of interference. It is defined as the ratio $R_{I,II}/R_I \cdot R_{II}$, where R_I and R_{II} are the fractions of particles with recombination in region I or II, respectively, regardless of possible other recombinations, and where $R_{I,II}$ is the fraction of double recombinants (see Fig. 1).

If this picture held true, the elementary mechanism of recombination has to be disentangled from complicated population phenomena. In this case, our first aim would have to be the complete understanding and the quantitative analysis of the population problem. If this problem could be solved, we would have improved our ability to draw conclusions concerning the elementary process.

THE ASSUMPTIONS

The above given general picture of phage multiplication and recombination shall now be the subject of a detailed discussion and of a quantitative analysis. Let us first see what set of assumptions is needed in order to get the mathematical formulation describing the above outline.

Assumption 1. In the very beginning we are confronted with the following fundamental question: Is the recombination mechanism between phages in one host cell one unitary, inseparable process or can it be reduced by the assumption of a population phenomenon (as described above) to a simpler basic subprocess occurring a number of times during phage development? The first alternative would indicate that the recombination mechanism in phage is of a very complicated nature offering almost no hope of being understandable. The second alternative, however, corresponding to the given outline, is in agreement with our general trend to simplicity and promises the possibility of a comparison of the basic process in phage with its analogue in higher organisms. This is the argument which led Visconti & Delbrück (25) to their first and most fundamental assumption: It is possible to break up the total recombination mechanism within one cell into a number of independent processes. "Independent" means that the recombining events in any given process will not be influenced by whatever happened or happens in all other processes. Different, but dependent recombining events are lumped into one and the same process. Each such process will be called a "cooperation." The term "mating," which was used by Visconti & Delbrück (25), describes a special model of such cooperation. The more general concept of cooperation, however, neither involves a definite number of participants nor of recombinants produced, nor breaks and reunions, nor any other specified assumptions. The term "cooperation," therefore, is given to any imaginable basic process leading to formation of recombinants. It is identical to the term "Serie" used earlier by Bresch & Starlinger (54).

The basic process of a cooperation should be clearly distinguished from the elementary act of recombination itself, which joins two pieces of genetic information derived from two distinct cooperating structures, recognizable by genetic markers or not. Following Hershey (55), this elementary act will be called a "switch of information source" or simply a "switch," again without any implications as to the possible mechanism involved. One cooperation may comprise zero, one, two, or more switches.

⁴ Hershey (55) and Steinberg & Stahl (56) continue to use the term "mating" for the generalized notion which we, in order to avoid confusion, propose to call a "cooperation."

Assumption 2. The second fundamental question we have to decide is the following: Can the offspring particles of a cross be the products of more than one cooperation? This question is not trivial, because one could imagine models in which a cooperation process terminated by maturation would prevent its produced structures from further cooperations. To achieve a simple mathematical picture we will, following Visconti & Delbrück (25), assume that cooperations are independent in their occurrence and can therefore be consecutive. It should be stressed, that Visconti and Delbrück had to assume consecutive matings because of the existence of triparental recombinants. This argument, however, holds true only if the genetic information of a recombinant emerging from a cooperation is never derived from more than two cooperating structures (as in their mating model). Later, this point will be discussed extensively. The increase of recombinant ratios during the latent period favors strongly the assumption of consecutive cooperations. The assumption, however, does not necessarily follow from this experimental fact, because the increase of recombinant ratios could arise from an increase of average recombination per cooperation.

Assumption 3. Connected with the randomness of cooperations is our third assumption, again introduced for the reason of mathematical sim-

plicity: the cooperation process is instantaneous.

The postulate of random cooperation processes can be reached also from a different viewpoint. Looking at the phage population resulting from a cross, we have seen that single switches are not randomly distributed. This is the phenomenon of negative interference. As one tends to keep a random distribution mechanism, one sets the assumption that "series of switches" rather than single switches are randomly distributed over the multiplying population of the vegetative pool, each series containing zero, one, or several single switches. This assumption is equivalent to our first assumption of independent cooperations, but implies, in addition, assumptions 2 and 3 and the later assumption 6. One series of switches in a particle is the result of one independent cooperation process.

Assumption 4. The limited number of particles infecting a cell leads to different infection ratios of genotypes in distinct cells. For mathematical simplicity we will, therefore, introduce the fourth assumption, namely: The cells will be regarded as infinite and equal. In other words, looking at the result of a mass lysate from a cross we will neglect all possible deviations arising from the fact that progeny phages come from finite and discrete cells. This, obviously, is in disagreement with the actual situation, but we are able to correct for the finiteness of cells afterwards, what will be discussed in a

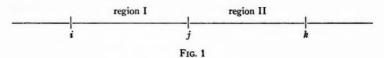
later section.

Assumption. 5. Likewise for simplicity, we will put forward the fifth assumption, that all carriers of genetic information, which are introduced into the cell by infection, will be reproduced and maturated with equal probability. Thus, the frequency ratios of any alleles, which have a certain value upon infection, will stay constant during the latent period, both in the pool of vegetative phages and among matured particles. This demands that

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there is no observable influence of special markers employed in a cross on their rate of reproduction and maturation. This assumption seems in general to be justified by the experimental data. However, several cases are known [e.g., Hausmann & Bresch (57)], in which a considerable influence of allelic markers can be seen.

Assumption 6. We still have to give three more rules to the cooperation process itself. For the sake of simplicity we will state the sixth assumption, that any structure in the pool shall have the same probability of contributing genetic information to a given recombinant structure emerging from a cooperation. This means that there is no influence of alleles or of the history of the contributing sources. Thus, neither their possibly past cooperations nor their number of reproductions must be relevant. It further demands that the mixing of structures in the pool is always ideal, i.e., there is no influence of the position of structures within the cell (no topography). The assumption, however, includes the case that only a randomly selected subpopulation can contribute to a given cooperation, for instance, by being in an activated special state or involved in a pairing.



Assumption 7. Beside this, one has to consider the different possibilities of recombination in one cooperation. For a 3-factor cross one introduces three independent probabilities per cooperation. Different sets of parameters are equivalent. Here, we will use p_I as the probability of a recombination between marker i and j, regardless whatever happens between j and k and, correspondingly, p_{II} for the recombination between j and k (see Fig. 1). Furthermore, $p_{I,II}$ will describe the probability that recombination occurs, both between i and j and between j and k. If there is no mutual influence between region I and region II (no interference), the so-called factor of coincidence $C = p_{I,II}/p_I \cdot p_{II}$ will be equal to unity. In general, however, recombinations in region I and region II will interfere $(C \neq 1)$.

One may ask whether all cooperations will have equal probabilities p_I , p_{II} , and $p_{I,II}$. To illustrate this point, let us imagine there would exist two kinds of cooperations with equal frequency, both without interference.

⁸ It should be distinguished between this negative interference of switches within the single cooperation process [called by Hershey (55) the "true negative interference"], which is measured by the factor $C = p_{I,II}/p_{I} \cdot p_{II}$ and between the negative interference among the total progeny population of a cross (see Footnote 1), measured by $C_{pop} = R_{I,II}/R_{I} \cdot R_{II}$. The latter originates partly from true negative interference, partly from the population phenomenon (random cooperation and maturation), and partly from deviations of this randomness. Details of this question may be seen in Hershey's paper (55).

The first kind shall have $p_I = p_{II} = 0.1$, $p_{I,II} = C \cdot p_I \cdot p_{II} = 0.01$; the second, $p_I = p_{II} = 0.3$, $p_{I,II} = 0.09$. If we look at the average p-values, we will find $p_I = p_{II} = 0.2$, $p_{I,II} = 0.05$. But this corresponds to a C-value of 1.25. A fluctuation of the p-values in single cooperations, therefore, would find its expression in a negative interference (C > 1). This shows that the question of equality of all cooperations is inseparably linked to the question of interference in a single cooperation.

Another point is the problem of the constancy of the average p-values during the latent period. If p_I, p_{II} , and $p_{I,II}$ change, but their ratios remain constant, no major difference in the formalism is needed, as can be seen from the mathematical analysis (54). Such a situation, however, is accompanied by a reciprocal change of the coincidence factor. If we state the seventh assumption: The probabilities of recombinations per single cooperation shall be constant during the latent period, we have included this case of a change in the p_I and p_{II} values with a corresponding reciprocal change in the coincidence factor C. But we do not include the possibility, that p_I and p_{II} are undergoing changes, while C remains constant.

Assumption 8. Our last assumption concerns the already mentioned question of whether the information of a recombinant structure emerging from a cooperation can be derived from two cooperating structures only or from more than two information sources. To make this clear, let us imagine that we could give numbers to all structures in the pool which carry information. Now, let us look at a product of one cooperation with two switches. If information can be contributed from only two sources, then these two (e.g., No. 7 and No. 12) have to alternate in the recombined structure as is indicated for one of two possibilities in Figure 2a.



Fig. 2

This case will be called a "pairwise cooperation." If, on the other hand, in one cooperation more than two information sources can contribute to a recombinant structure, let us say, No. 7, No. 12, and No. 5, then one possible recombined structure corresponds to Figure 2b, and we will refer to this case as a "group cooperation." Such a group cooperation may be "complete" or "incomplete." If there is a random contribution of pieces of information from any information source in the pool, the ideal case of complete cooperation is realized. If, however, the probability of deriving two pieces of information from one and the same contributing structure is higher than expected from randomness, this case will be referred to as incomplete cooperation.

⁶ A "piece of information" extends over the distance from one switch to another.

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Now, we can state our eighth and last assumption, namely: The degree of completeness of the cooperation shall be constant within the latent period.

THE PARAMETERS

In higher organisms the genetic composition of the products of a meiosis can (in diploids by means of an appropriate backcross) be investigated directly. The parameters of this process, i.e., the probabilities of the different possible recombinations, are therefore directly given by the frequencies

of the corresponding genotypes in the daughter generation.

In the genetics of phage we used the same parameters p_I , p_{II} , and C to describe the meiosis analogue, namely, the basic process of a cooperation. The numerical values of these parameters, however, are not identical to their corresponding genotype frequencies in the yield of a cross (as in higher organisms), because those are, in addition, determined by the frequency of cooperations, by the conditions of the infection, and by another parameter Q, accounting for the completeness of cooperations. We will see that the possibility of disentangling these factors is rather limited. Before we give the result of the mathematical analysis, we have still to decide exactly in what measure we want to account for the frequency of cooperations. The mathematical treatment suggests the introduction of a "cooperation experience," which is the generalized "mating experience" of Visconti & Delbrück (25). To understand its significance, let us consider some details. We say that upon infection all structures in the pool start with a cooperation experience M=0. Now, if a cooperation takes place, we ascribe to all emerging structures which, by means of the unknown mechanism, could be recombined structures, i.e., to all "potential" recombinants, the cooperation experience M=1 (regardless of whether their information is really derived from two or more distinct sources and whether or not existing switches can be recognized from the genetic markers employed). The experience M=1 will further be ascribed to all future structures, which will receive their information from such a potential recombinant without further cooperations. If, in such a later cooperation, a new recombinant structure is formed which derives its information from sources already carrying the experience M=1, it will have an increased experience of M=2. Obviously, we run into a difficulty, if sources with different experiences contribute information to a new potential recombinant. What experience should be given to such structures? One can ascribe to it a nonintegral M-value according to the lengths of information pieces and the experiences of their corresponding sources. This was done by Bresch & Starlinger (54). Another, in a certain sense, better way, proposed by Stahl & Steinberg (56), follows the "line of descent" of an arbitrary marker to one of the information sources. This one then will "bequeath" its experience to the entire structure. The advantage of this definition lies in the integer M-values of all structures. Since, in fact, the experience of a single particle has no meaning to the analysis, nor can it ever be determined experimentally, both definitions are equivalent leading to the same average cooperation experience of the phage population which will be used as the parameter of the cooperation frequency.

Beside this, another question can be treated in slightly different ways. To what structures involved in a cooperation should be ascribed the experience of this cooperation? Steinberg & Stahl (56) give the experience to all structures involved, including potential recombinants and the contributing information sources. Then they multiply the average M-value by a (not necessarily constant) factor $F \leq 1$, which corresponds to the fraction of all structures involved in a cooperation process, which emerge as potential recombinants. On the other hand, Bresch & Starlinger (54) ascribe the cooperation experience (as we did here) to potential recombinants only. This difference in the treatment, again, has no significance. But, as the parameters M and F can never be separated, we will stay with the second alternative. The so-defined average cooperation experience M is identical to the product m. F used by Steinberg & Stahl (56). In the Visconti-Delbrück theory (25) this question does not arise, because in this specialized model (as in all breakreunion models) all particles involved in a mating can emerge from it as recombinants (F=1).

The last parameter to be considered is the completeness of cooperation. As can be understood from the mathematical analysis only, it is appropriate to ascribe a value of Q=1 to the ideal case of complete cooperation, while pairwise cooperation corresponds to Q=2, and different degrees of incompleteness to 1 < Q < 2. Thus, (2-Q) measures the fraction of all cooperations with recombination in region I and region II, in which the three markers i, j, and k are contributed from three distinct sources. It should be stressed, however, that the value of the parameter Q is attributed to a special triplet of markers like p_I , p_{II} , and C and is not an a priori constant of a given virushost-system like M.

THE ANALYSIS AND ITS RESULTS

Before we discuss the analysis itself let us briefly summarize the used assumptions: With two fundamental assumptions, we introduced consecutive (assumption 2), independent cooperation-processes each involving zero, one, or more switches (assumption 1). All remaining assumptions set up simplifying rules for these cooperations in order to get a mathematically treatable picture. They stated instantaneous cooperations (assumption 3); infinite and equal cells (assumption 4); equal probabilities of all information carriers to reproduce and to mature (assumption 5), and to cooperate (assumption 6); and, finally, during the latent period constant features of the average cooperation process, described by the constant parameters p_I , p_{II} , C (assumption 7), and Q (assumption 8).

We will use the following parameters: (a) The average cooperation experience of the population M, intrinsic to the host-virus-system employed. (b) The probabilities of recombination p_I , p_{II} , the factor of coincidence C, and the factor of completeness Q of the single cooperation process, all intrin-

sic to the employed triplet of genetic markers. (c) The relative frequencies of infecting genotypes, intrinsic to a given experiment.

The first and basic analysis of phage population genetics following these lines was given by Visconti & Delbrück in 1953 (25). Their picture of the cooperation mechanism, however, was more specific than was necessary. The explicit discussion given here differs from their "mating theory" essentially only by the introduction of the factor of completeness. Visconti and Delbrück considered only the case of pairwise mating. The possible alternative, namely, the complete cooperation and the synthesis of both into the most general case of incomplete cooperation was realized but recently by two groups of phage geneticists independently of each other. Steinberg and Stahl, stimulated by Hershey's work on the multiplicity reactivation of T-even phages after ultraviolet treatment (55), set up the generalized equation by a statistical method (56), while identical results were obtained by Bresch, Starlinger and Hausmann (54, 57), using differential equations. This latter group was led to the problem by the experimental attempt to decide between the model of a pairwise mating (Visconti-Delbrück model) and the partial replica hypothesis (58), which is a representative of a complete cooperation mechanism (see the later section on models). The experiments of both groups will be reviewed in a later paragraph. In both mathematical methods the obtained equations express the frequencies aik of the different genotypes ijk resulting from any 3-factor cross as functions of the aforementioned parameters:

$$\begin{split} a_{ijk} &= A + (B-B_i) \cdot e^{-Mp_I - Mp_{II} + Mp_I \cdot Mp_{II} \cdot C/M} \\ &+ B_i \cdot e^{-Mp_{II}} \\ &+ B_k \cdot e^{-Mp_I} \\ &+ B_i \cdot e^{-Mp_I - Mp_{II} + Mp_I \cdot Mp_{II} \cdot C/M \cdot Q} \end{split}$$
 Equation 1.

where

$$A = a_{i,.}a_{.j,a_{..k}}$$

$$B = a_{ijk}(o) - a_{i,.}a_{.jk}(o) - a_{..k}a_{ij.}(o) + A$$

$$B_i = a_{i,.}a_{.jk}(o) - A$$

$$B_j = a_{.j.}a_{.jk}(o) - A$$

$$B_k = a_{..k}a_{ij.}(o) - A$$

are the constants of the initial conditions, in general different for each genotype. Details of that formula and the mathematical way to reach it, may be seen from the original papers [Steinberg & Stahl (56); Bresch & Starlinger (54); Hausmann & Bresch (57)].

The proper problem to be solved, however, is the inverse, namely, the numerical determination of the parameters M, p_I , p_{II} , C, and Q from the experimental results, i.e., from the genotype frequencies observed. We thus have to invert the formulas and to describe M and p_I , p_{II} , C, and Q as functions of the resulting genotype frequencies a_{ijk} and of the constants of the initial conditions. But, from the given equation 1 it can be seen, that M, p_I ,

 p_{II} , and C never appear but in the combinations Mp_I , Mp_{II} , and M/C. Therefore, only these combinations can be determined numerically and never single values of M, p_I , p_{II} , or C. Thus, we cannot separate the single event from the population genetics or, in other words, we are not able to distinguish between many cooperations with a high factor of coincidence and small recombination probabilities on one hand, and few cooperations with a small C and high p-values on the other hand.

In all 2-factor and all 3-factor crosses with symmetrical infection by only two parental types the term $(B-B_j)$ in Equation 1 will be zero for all genotypes. Thus, from such crosses the combination M/QC is the only numerically determinable value, i.e., one cannot even separate M/C and Q. On the other hand, Q can be calculated from 3-factor crosses with 3 parents or

with asymmetrical infection by 2 parents.

The fact that no single value of the parameters M, p_I , p_{II} , and C can ever be obtained, is fundamental to the population problem. To demonstrate this point let us consider a group of 2-factor crosses with increasing marker distances. In this group an increase of recombinant frequencies is found up to a certain maximal limit. Markers in this saturation distance are regarded as unlinked. In a 3-factor cross involving a saturated distance ij (see Fig. 1), the recombination probabilities per cooperation between i and j, namely $p_{sat} = p_I$ and between i and k, namely $[(p_I - Cp_Ip_{II}) + (p_{II} - Cp_Ip_{II})]$ are equal. [For simplicity we have assumed the condition of pairwise cooperation (Q=2).] From this follows $2Cp_{sat} = 1$ or more general $QCp_{sat} = 1$. In this determination the value of p_{II} , corresponding to the extension of region II, is of no importance. This means that any triplet, in which region I or II or both are saturated, has the same value of the product $(QC)_{sat}$.

To decide the question whether we have a priori knowledge of the numerical values of p_{sat} or $(QC)_{sat}$ let us consider two different possibilities of the origin of saturation: (a) If, in the cooperation process, switches are randomly distributed over the entire structure [no interference, C=1], saturation can only be obtained for a distance great enough to contain an odd or even number of switches with equal probabilities. (This is reached exactly only for an infinite interval, but the approximation is already rather good with an average switch number of two). On the other hand (b), saturation can be obtained with a smaller number of average switches, if negative interference is involved. Then the switches are not randomly distributed, but in such a manner that though an extension of the distance would bring in more switches, exactly half of these new switches would meet an odd, the other half an even number in the original interval. Both alternatives (a and b) are possible explanations of the saturation observed in phage crosses. Therefore, we have no a priori knowledge of numerical values neither of (OC)_{eat} nor of p_{sat} , but only of the equation:

 $p_{sat} \cdot (QC)_{sat} = 1$

Equation 2.

which is valid for all crosses with so-called unlinked markers. If, in a given

phage, negative interference in the single cooperation can be shown experimentally for small intervals (we will discuss such case a little later), we can be sure that C_{sat}>1, because a nonrandom distribution of switches in a small interval does not become random by extending the interval.

As p_{sat} is unknown we cannot even by the employment of "unlinked" markers determine the value of any single parameter. But we can give some limits of their numerical values: Since p is a probability and can never exceed unity, we can rule out, from the existence of markers at a saturated distance, the possibility of strong positive interference $[(QC)_{sat} \ge 1]$. Markers reaching saturation are found in all well-investigated phages [T2: Hershey & Rotman (7); T4: Doermann & Hill (10); T1: Mennigmann (18); λ : Kaiser (14)]. An upper limit for C cannot be given. On the other hand, likewise a minimal M is ascertainable from the experimental values of pM.

Since, apart from these minimal limits of M and C_{sat} , the determination of single parameters is in principle impossible, the M-values frequently cited in the literature (usually called "rounds of mating"), which are all calculated under the by no means justified assumptions of no interference (C=1) and pairwise cooperation (Q=2), equal, in fact, not M, but rather $2M/(QC)_{sat}$. Therefore, strong caution has to be applied to all speculations and conclusions which are based on the M-values of certain phages which are thus "determined." The frequently found statement, that T1 or λ has a smaller cooperation experience ("rounds of mating") than T2 or T4, is rather senseless. With the same right one could claim that the C-values were higher in T1 or λ than in T-even (assuming equal M and O values).

Let us now consider the question of high negative interference (36). Could we not perhaps overcome the fundamental difficulty of determining single values of M, p_I , p_{II} , and C by employing a triplet of very closely linked markers? In such a case the "effective" cooperations (those giving rise to observable recombinants) will be very rare. Therefore, one could think that the factor of coincidence of the interference among the population of offspring particles (C_{pop}) would approximate the factor of coincidence C of the single cooperation. In calculating the population interference (C_{pop}) from Equation 1 we find, however, for closely linked triplets and symmetrical infection with 2 parental genotypes 111 and 222 (replacing e^{-pM} by 1-pM),

$$C_{pop} = \frac{a_{121} + a_{212}}{(a_{12} + a_{21}) \cdot (a_{.12} + a_{.21})} = \frac{QC}{M}$$
 Equation 3.

showing that also here no single parameter can be measured. Chase & Doermann (36) performed many such experiments in T4. They observed over a relatively large range of marker distances (excluding the extremely linked triplets), values of C_{pop} around 6. Concerning unlinked markers in T4 we know from Doermann & Hill (10), that $2M/(QC)_{sat} = 5$ or $(QC)_{sat}/M = 0.4$. Though we are not able to give an absolute value of any C, we can (assuming the same M and Q for all T4 crosses) state, that the factor of coincidence for closely linked triplets is about 15 times higher than for unlinked triplets.

It is, however, perhaps more reasonable to assume $Q_{\rm sat}=1$ (complete cooperation) for unlinked and Q=2 (pairwise cooperation) for closely linked triplets. Then, the value of the coincidence factor of the investigated closely linked triplets would be 7.5 times higher than for unlinked triplets.

In summary, what we find from experimental genotype frequencies by application of the mathematical analysis are only relative values of p_I, p_{II} , and 1/C. The value of M remains a free proportional constant. This shows that the concept of our analysis accomplished by the set of assumptions given above, contained one surplus parameter. With a simpler set of assumptions and parameters one should be able to reach the same mathematical solution. We will return to that basic point in the last section.

THE COMPLICATIONS

It is not the purpose of the following section to discuss all possible deviations from the original set of assumptions, but rather to consider a few obvious complications and difficulties to the analysis given.

Spread in maturation time.—According to assumption 5, vegetative particles will be withdrawn from the pool randomly in order to be matured into infective phage. As this process takes place in all of the second half of the latent period, infective progeny particles leave the pool at different values of its average cooperation experience. If the spread in maturation time shall be taken into account, one obtains slightly more complicated formulas for $p_I M$, $p_{II} M$, and M/C. For this correction one must assume that the maturation rate per unit increase of the cooperation experience in the pool is constant. This additional assumption, however, can well be justified by its agreement with premature lysis data [T4: Doermann (9); T1: Bresch & Trautner (17)]. The mathematical treatment then introduces only one additional parameter, describing the fraction of the cooperation experience of the pool reached at the time of the onset of maturation, compared to its value at lysis. The value of this parameter, however, can be taken from premature lysis experiments. The corresponding mathematical treatment has been carried out by Visconti & Delbrück (25). Recently it has been developed in more detail and set into a formalism adapted to routine calculations by Hausmann & Bresch (57).

Finiteness of cells.—As the bacteria, to which phages adsorb, are finite, only a limited number of random particles will infect each cell. Therefore, the initial ratios of genotype frequencies will vary in different cells (compare assumption 4). This point has been taken into account by Lennox, Levinthal & Smith (59), who gave a correction formula to genotype frequencies resulting from a cross for different average multiplicities of infection. Although their calculation is based on equally sized bacteria and for this reason is only an approximation, the effect of the limited infection is well understood in principle. As cooperation is assumed to be instantaneous there can be no other influence from the finiteness of host cells.

Inhomogeneity of cells. Our fourth assumption included, on the other

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hand, the equality of all host cells. The focus of interest here is less the size of bacteria or their burst size (number of phages from one cell) but rather the average cooperation experiences in yields from individual cells. We do not know whether their fluctuation corresponds to a random appearance of cooperations or whether the bacteria consist of subpopulations having for physiological reasons a priori different probabilities concerning the occurrence of cooperations. Calculations of this kind would not offer any principal difficulty, but they cannot be carried out as long as the distribution function is unknown.

Finite cooperation time.—The assumptions 1, 2, 3, and 6 lead to a Poissonian distribution of the cooperation experience among single particles in the pool. This does not, however, demand a constancy of the probability of cooperations during the latent period, which is suggested by experimental data [Levinthal & Visconti (52); Bresch & Trautner (17)]. But a constant, noninfinitesimal duration of the cooperation process would disturb the Poisson distribution in the direction of a smaller variance. It would approach (as Steinberg pointed out in personal discussions) the alternative case of a uniform experience of all particles in the pool, which is realized if the time spent in a cooperation is very long compared to the probability per information source of entering a cooperation process. This case has been considered and analyzed mathematically by Visconti & Delbrück (25) under the term of "synchronous mating." A deviation from the third assumption of instantaneous cooperation would thus approach their corresponding formulas. Visconti and Delbrück, however, could rule out the extreme case of "synchronous mating" by their experimental data, which agreed much better with a "random-in-time mating," equivalent to the instantenous cooperations in our terminology. Nevertheless, the assumption of an instantaneous process is an artificial picture, which can at best be well approximated. But even if we cannot yet quantitatively account for a possible deviation, we know that the correction tends towards the result of "synchronous-mating" formulas (25).

The multiplicity effect.—Recently, a phenomenon was observed by Trautner & Bresch (60), which indicates that another correction will be necessary, at least for certain phages. Let us compare the recombinant frequencies of several crosses with increasing average multiplicities of infection. The theory predicts (after correction for finite cell size) no influence or perhaps a slight decrease, because of the smaller multiplication per infecting particle with increasing multiplicities. In disagreement with this expectation, a rather strong increase of recombinants was found in T1 and P22, the only phages tested up to now. Premature lysis experiments further show that this increase can be observed not only in the first part of the latent period, but to a somewhat smaller extent, also shortly before lysis. This unexpected phenomenon may be partly responsible for the well-known difficulty to reproduce quantitatively data of cross experiments, especially in phages of a sensitive and weak adsorption mechanism. The explanation of this multi-

plicity effect has not yet been found. Two possibilities, however, are favored, both rather discouraging to the attempted quantitative analysis of population genetics: The first explanation would be the existence of areas of single genotypes within the host cells. These could arise from the tendency of daughter particles to stick together after multiplication. Such a "topography" would create an influence of the local positions of single information sources within the host cell on the probability of contributing to a given cooperation. This would contradict our sixth assumption and would reduce the value of the mathematical treatment.

As another explanation, a "waiting-room" model has been proposed by Steinberg in a personal discussion. The essence of that idea is the following: after infection all entering information sources are assembled within a waiting-room and there prevented from beginning multiplication and cooperation. After a certain time, the waiting-room is opened and the single particles may escape into the pool of multiplication and cooperation. If one assumes, for instance, that the probability of leaving the waiting-room is equal for all collected particles and constant in time, the multiplicity effect could be understood. The waiting-room idea is supported by some experimental results in animal viruses [Cairns (61)]. As Steinberg pointed out, it should be testable by a statistical analysis of gene frequencies in single bursts. Whatever the explanation may be, the analysis of the population genetics demands an additional correction similar to that of final cell size, which can be given quantitatively only after more details of the observed phenomenon are settled.

The influence of markers.—It is possible that the presence of certain alleles in a structure will have an influence on its statistical behavior in the pool. One can distinguish three kinds of such influence. The first will be reflected in differences in the probabilities of some genotypes to multiply or to mature. As already mentioned, such cases are known [e.g., (57)]. Many mutants show selective disadvantages compared to the wildtype as can be seen from the asymmetry of the number of output genotypes in a mass cross in spite of a symmetrical infection. Nevertheless, these physiological differences between certain genotypes, being troublesome to geneticists from the technical viewpoint and perhaps interesting to physiologists, are rather irrelevant to the proper problem of population genetics in phage, as markers can always be found which show only little or no effect of this kind.

There may be, however, a second influence of alleles, namely, on the probability of recombination, i.e., either on the probability of cooperation or on the parameters of the single cooperation. Such a situation can never be recognized from one 3-factor, 2-parental cross only. To check it, we have to compare the recombination frequencies between two given markers in different crosses, employing various markers as the third. In most of such cases, a satisfying agreement in the recombination between the pair of test markers is found. Therefore, occasional differences are usually ascribed to the general fluctuation of biological experiments. Nevertheless, some phage

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geneticists have suspected, during the last years that alleles might influence the recombination between two given markers.

This rather specific influence of alleles should be distinguished from a third kind, called by Hershey "marker stimulation" (55). This hypothesis assumes that recombination is favored by a heterogeneity of structures, i.e., not the employed alleles themselves, but the dissimilarities in genetic information of the parents will account for the probability of cooperation or of recombination per cooperation. To make this difference clear, let us consider the results of three crosses: $A \times B$ has a certain percentage of recombinants (r per cent). $AB \times C$ or $AC \times B$ or $A \times BC$ may show a higher percentage of recombinants between A and B. Now, in the cross $AC \times BC$ the recombinant percentage has to drop again to about the value of r per cent, if the heterogeneity influences recombination (marker stimulation), but the percentage can correspond to that of $AC \times B$ or be still higher, if the C allele itself is responsible for the increase (allele-influenced recombination). For the time being, this complex problem cannot be solved. The existence of "allele-influenced recombination" or of "marker stimulation" in phage and perhaps in higher organisms should be the subject of future investigations.

EXPERIMENTS ON THE COMPLETENESS OF COOPERATION

One of the three available experimental approaches to this question is Hershey's recent study (55) of active phages coming from multiplicity reactivation after heavy ultraviolet irradiation, given to cells shortly after their symmetrical infection with two parental types, ab^+ and a^+b (a and b linked). The critical point here is the frequency of different genotypes among the multiplicity-reactivated offspring. According to earlier hypotheses [Luria (62)] and recent experiments of Epstein (63), multiplicity reactivation is the result of a mechanism similar to genetic recombination, by which undamaged pieces of information from different inactivated parental structures are contributed to a new active particle. If this cooperation process between the ultraviolet irradiated parental structures were complete, distant genes (i.e., genes separated by at least one ultraviolet hit) of the emerging active particle would be derived from random information sources in the pool. As all active particles would originate from such cooperations, one should find genetic equilibrium in the yield (each genotype 25 per cent). On the other hand, if the cooperation process were always pairwise, half of the cooperations would occur between two genetically identical structures for statistical reasons. From these, only active parental genotypes can emerge. The other half of the cooperations would occur between two genetically different structures and would give origin to all four possible genotypes with equal probabilities. To sum up, from the reactivating cooperations we may expect in a pairwise process a 1:3 ratio of recombinants to parental types, but a 1:1 ratio in the case of complete cooperation.

The result, obtained by Hershey, is more consistent with the assumption of complete cooperation. Nevertheless, as Hershey pointed out, some objec-

tions can be made against a generalized conclusion which arises from this fact. First, the result of the original reactivating cooperation processes could be obscured by later "marker rescue" in cooperations between active and inactive structures. But this can be tested by an analysis of individual bursts, which—as Epstein (63) found—show a tendency, increasing with the ultraviolet dose applied, to consist of a strong majority of one genotype. This indicates that we find in general in each cell the result of one random reactivating cooperation only. With high ultraviolet doses, therefore, the recombinant percentages from a multiplicity-reactivating mass cross do approximate the average result of the original reactivating cooperations.

On the other hand, it is conceivable that large numbers of inactive recombinants result from cooperations among inactive parental structures before the reactivating cooperation occurs. Finally, if this could be ruled out, still the essential argument is left, that cooperations among ultraviolet-inacti-

vated structures may be different from the normal mechanism.

Another kind of experiment to determine the completeness of cooperation was recently performed in T1 by Hausmann & Bresch (57). It was thought that the large number of distinguishable genotypes (even among allelic markers) in this phage would allow a determination of the value of Q. Therefore, a symmetric 3-allele-3-factor-3-parental cross of the type 121×212 ×223 was performed. In the case of complete cooperation the triparental double recombinant 113 should be produced with the same frequency as the biparental double 111. On the other hand, if cooperation is pairwise or incomplete, the biparental recombinant 111 should be more frequent. In spite of technical difficulties a reliable result was obtained in agreement with incomplete cooperation. The result, however, could also fit an obligatorily pairwise cooperation, if cooperations would occur between nonrandom partners. As shown in the section on complications, there is reason enough to believe in such argument (see the multiplicity effect). Therefore, no final conclusion can be drawn on the basis of this experiment.

As a third at least partially successful approach, the experiments of Edgar & Steinberg (64) should be discussed where, in a biparental cross 111×222 (T4, strongly linked triplet), the frequencies of the double recombinant 121 are measured over a large range of different input ratios of the two parents. If this ratio is $\alpha:(1-\alpha)$, it follows from Equation 1 applying the approximation for small pM-values:

$$\frac{a_{121}}{Mp_I \cdot Mp_{II}(\alpha - \alpha^2)} = \frac{C(Q-1)}{M} + \frac{C(2-Q)}{M} \cdot \alpha \qquad \qquad \text{Equation 4} \, .$$

The different values of the left side of this equation can be calculated from the experimental data given by Edgar & Steinberg (64) and can be plotted against α . From this diagram one obtains values of C (Q-1)/M and C (2-Q)/M, from which rough values of Q=1.8 and C/M=7.1 are found. Nevertheless, because of the inevitable fluctuation of the data, the experiment would be consistent also with a value of Q=2. The result shows

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that in strongly linked triplets of T4 the pairwise cooperation is at least well approximated. But, also, here a possible existence of a topography would influence the result.

In summarizing the three kinds of experiments it must be stated that in spite of all efforts the fundamental question of the completeness of cooperation concerning loosely linked or "unlinked" triplets is left entirely open.

CATEGORIES OF MODELS

Recombination in phage has been explained by a variety of models and submodels. Some of these are intermediates between different groups, others, looking very different, are formally identical. Here, we will neither consider all details of current conceptions nor exhaust all possibilities of models; rather, we will—following Hershey (55)—confine ourselves to some essential features and contrast these to the set of assumptions involved in the given analysis.

Let us start with the idea of a switch. This is a point in the supposed linear structure [Benzer (47); Chase & Doermann (36)] of a phage genome, at which two pieces of information from different sources are connected. How can such a switch arise? In principle, there are three possibilities: (A) Both pieces of information forming the switch are pre-existent and are glued together, or (B_1) only one of the two pieces of information pre-exists, the other is synthesized de novo as a continuation of the first piece, or (B_2) neither one is pre-existent. Then the switch becomes the starting point of a de novo synthesis in two directions. In case (A) recombination would be independent of reproduction, whereas (B_1) and (B_2) connect both these phenomena intimately. We will refer to group (A) as the "break-reunion group" of models and to the (B) cases as the "copy choice group" [term introduced by Lederberg (65)].

On the other hand, models of phage recombination can be divided by another criterion. (I) In the first group of "unitary models" (term suggested by Steinberg in personal discussion) cooperations occur between carriers of complete genetic information only, and all end products of cooperations will again be structures of complete information. (II) The group of "fracturing models," on the contrary, involves fragments of information as essential to

the cooperation mechanism.

We can, furthermore, apply the completeness of cooperation as a third crucial feature by making a division into (a) models with pairwise cooperation and (b) models with group cooperation. By means of these characteristics one can classify each model into one of the eight categories obtained. The classical model of Visconti & Delbrück (25), for instance, implying pairing (mating) of complete vegetative particles followed by breaks and reciprocal reunions, falls into group AIa, whereas its old counterpart, the partial replica hypothesis (58) consisting of the independent building of partial genomes, which are later randomly assembled, belongs to AIIb. All eight categories are in accordance with the analysis given, i.e., no group of models is a priori

excluded by our set of assumptions. Experimental data, however, can decide the completeness of cooperations and thus rule out either (a) or (b). Unfortunately, as we saw, unambiguous data are not yet available. It should be mentioned, perhaps, that a group rather than a pairwise cooperation mechanism would resemble the meiosis of higher organisms, in which the cytological pictures of polyploides indicate a corresponding behavior of chromosomes. Neither of the other alternatives (A or B, I or II) can ever be finally decided by cross experiments alone, because all four types are described by the same mathematical formalism. Though no category of our scheme is excluded as a whole from the mathematical analysis, special submodels of any category can contradict the assumptions made. There seems to be little sense in a further discussion of special models, as long as no crucial experiment can be suggested to decide their validity.

FACTS AND FAITH

We started the analysis of population genetics in phage with the convincing concept of consecutive and well-defined cooperation processes. Then we saw that first the cooperation experience of a single particle and later even the average cooperation experience of the population turned out to be an indeterminable and senseless idea. This could be seen from the impossibility of ever determining absolute values of p_I, p_{II}, C, and M. The deeper reason for this calamity lies in our basic concept, which attempted to describe more than is justified. How could we ever hope to measure how many cooperations will occur with no switches? By the introduction of the parameter C we accounted for the relation of the occurrences of one and two switches, but the number of cooperations with zero switches was left open. Nevertheless, in our picture they strongly contribute to the average experience of the pool. We thus have one surplus degree of freedom in our concept, because we are not dealing with unitary, well-defined, all-or-none cooperations, each with constant recombination probabilities. As already shown in the discussion of assumption 7, the question of equality of all cooperations is inseparably linked to the factor of coincidence. These are identical problems. The entire concept of well-defined cooperation processes, thus, fades away. Therefore, we can ascribe to the parameter M an arbitrary, but reasonable value. Here, immediately, the number of generations is suggested, if one has a copy-choice mechanism of a unitary type in mind. In the case of breaks and reunions, no equally meaningful assumption can be made. But let us consider a unitary copy-choice model and follow this line a little further.

The "cooperation" process will now be conceived as the building of a new replica, which may happen under the control of one structure only (this we are now obliged to call a "cooperation" with zero switches). If the replica is formed by a "cooperation" of two (paired) structures, switches can take place. If, further, the pairing would extend over a certain region of the structures only and would not cover the entire length of the phage genomes, we can explain the phenomenon of high negative interference by the diversity of

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single cooperations. This idea was originally introduced by Pritchard (66) concerning Aspergillus and applied to phages by Chase & Doermann (36). Such partial pairings between several structures are in agreement with a Q-value of about 2 for strongly linked triplets, as could be calculated from the experimental data of Edgar & Steinberg (64), (see Equation 4), though group cooperation (1 < Q < 2) is expected for loosely linked markers. This partial pairing hypothesis clearly illustrates that the concept of a well-defined, all-or-none cooperation is not justified. How could we give the same weight to a "cooperation" involving a pairing of a considerable length and to another with a very short (time and space) pairing? Where is the limit; what is to be called a "cooperation" and what is not?

Let us return to the suggested identification of cooperation experience with the number of generations. Under this assumption the M-value should vary in a certain range according to the number of infecting particles and the burst size. In T1 (and P22) an effect in the direction opposite to expectation is observed (60), which could be explained, for instance, by a topography. In T-even phages, however, such a phenomenon should perhaps be observable, if it is not compensated also by a slight influence of topography. It cannot be decided yet, whether the general difference between the "integral recombination probabilities" (pM-values) in T1 and λ on one hand, and in T-even on the other, may be explained by such secondary effects as, e.g., topography, or by intrinsic differences in their genetic structures. Biochemical studies showed such differences (67, 68, 69).

The general correctness of this picture would hold true even if the future brings the mentioned complications into the foreground. In such a case, we would have to confine ourselves to still worse approximated values and calculations than we now use.

In these considerations we could omit the existence of partial heterozygotes [Hershey & Chase (27); Levinthal (31); Edgar (37, 38); and Trautner (70)]. Apparently, they play no essential role in the formal population genetics of bacteriophage. Their importance, however, cannot be overestimated when one tries to build a bridge between purely genetic data and the structure of DNA, which is the ultimate aim of all efforts. Phage, as a genetic system, has a unique position relative to this future program.

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NEW VIRUSES AND VIRUS DISEASES OF MAN1,2

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INTRODUCTION

Expressions concerning the recent revolutionary advances in virology have been so often repeated that they are now trite. Nevertheless, the veteran worker must marvel at the remarkable contrast of present-day virology compared to that of 15 or more years ago. The isolation of a virus, once cumbersome, tedious, and expensive, and consequently a noteworthy feat, has now become a commonplace procedure. The development of new techniques for the cultivation and quantitative examination of viruses permits deeper probing into their intrinsic properties and their association with diseases. This has fired the imagination of novitiates as well as experienced workers, and has attracted individuals from allied sciences to this field, previously shunned because of its cruder methods.

The application of tissue culture methods and plaque techniques has placed the virologist today in a position similar to that of the bacteriologist about 75 years ago. However, he has surpassed the potential capability of the bacteriologist of those times and, in certain respects, even that of his contemporaries. Utilizing the new tools, virologists have been able to isolate at least 150 previously undiscovered agents in a matter of a few years. These "new" viruses have been encountered at such a rapid rate that they have presented new and difficult problems with respect to their status within the microcosm, their ecologic interplay, and their role as causes of disease (45, 46). A large number of the new viruses inhabit the environs of the gastrointestinal tract, about half are included among arthropod-borne viruses, and at least one-fourth may be responsible for recently differentiated respiratory diseases. The isolation of new viruses has also stimulated renewed active interest in the viral etiology of tumors (25, 31, 91).

It is reasonable to expect that the sudden presentation of an array of new agents would clarify previously unanswered questions in virology and at the same time tend to alter existing concepts as well as to create new and intriguing problems. Despite the overwhelming list of newly isolated viruses and their association with some disease entities, it remains peculiarly true that the majority of respiratory, central nervous system, and gastrointestinal disturbances of man cannot be attributed to the currently described in-

¹ The survey of the literature pertaining to this review was concluded in January, 1959.

² The following abbreviations will be used: ECHO (enteric cytopathogenic human orphan); ECMO (enteric cytopathogenic monkey orphan); ECBO (enteric cytopathogenic bovine orphan); ECSO (enteric cytopathogenic swine orphan).

fectious agents alone. The etiology of approximately 50 to 80 per cent of the infectious diseases of these systems still remains undetermined.

In order to review broadly the present status of this vast subject, an attempt will be made to examine some of the highlights of current developments within three major virus disease groups and to assess the impact of recent observations in the study of virus diseases.

ENTEROVIRUSES

The intense interest and laboratory activities concerning poliomyelitis and the polioviruses have resulted in the accumulation of voluminous literature relative to the properties of viruses in general, their behavior in man, and some of the factors predisposing to disease. Improvements in techniques have led to the isolation of additional virus strains which commonly inhabit the gastrointestinal tract. The enterovirus group (17), composed of more than 50 antigenically distinct strains of Coxsackie, ECHO (enteric cytopathogenic human orphan) (16), and poliomyelitis viruses has presented new and challenging problems in areas of classification, epidemiology, and etiology of infection (58). Enteric cytopathogenic orphan viruses have been recovered also in fair numbers from monkeys, bovines, and swine. These have been labeled ECMO, ECBO, and ECSO viruses by Melnick (56).

Paramount among the problems associated with the enterovirus group has been the inadequacy of the original criteria used for differentiation (20) and the lack of a satisfactory system of classification. The isolation and grouping of Coxsackie viruses were determined by the degenerative changes produced in suckling mice (18, 19, 54), the ECHO viruses were characterized by cytopathogenic effects in monkey kidney tissue culture (56), while polioviruses were demonstrated by the paralytic effect in monkeys. Transient existence in the human alimentary tract appeared to be the only common property binding these agents into a generic group (33, 44).

A second factor causing confusion in the classification of the enteric viruses is the high degree of adaptability or mutability exhibited by some of these agents. The properties of several strains of the enterovirus group appear to differ with varying passage histories, even revealing differences between strains of the same antigenic type. This may account for differences observed between freshly isolated and prototype strains, and variation in experimental manipulations may explain discrepancies of results from various laboratories (19, 55, 92). These observations serve to emphasize the fact that a classification system based on tissue tropisms and animal susceptibilities is unsuitable and at best useful only as a temporary expedient.

The antigenic interrelationships among the subtypes of enteroviruses are confusing at present but further studies on the basic antigenic structure of these viruses may eventually provide a more logical means of classification. An example is the situation with ECHO type 6 where the prototype strain ECHO 6 gives one-way serologic crossing with the related strains ECHO 6 prime and 6 double prime. The rather puzzling reports of antigenic

similarity between ECHO virus types 1 and 13 may be explained by recent findings suggesting a possible mixture of the two types in early studies (56). Cross neutralization is also observed between ECHO types 1 and 12 and between types 1 and 8 (17, 56).

Among the polioviruses, physical or chemical inactivation appears to uncover a broader spectrum of complement-fixing antigens, as evidenced by serologic cross reactions among the three types (57, 82). This is in contrast to the type specificity displayed by active viruses against neutralizing antibody. The neutralization test measures the infection-blocking properties of a serum and appears to involve only a portion of the antigenic structure of a virus particle. From studies with bacteriophages and the Newcastle disease virus it is deduced that only a limited area on the virus surface must make effective contact with the susceptible host cell surface to achieve infection. Since, after absorption of the neutralizing activity from immune sera, the complement-fixing antibody still remains, it is concluded that complement-fixing antibody results from the antigenic stimulus of areas on the virus surface which are probably not associated with infectivity (29, 76). A complement-fixing antigen which has one-half or one-third the diameter of the infectious particle has been reported for several ECHO viruses (56). If this proves significant, it may lead to the development of a rapid diagnostic procedure and possibly provide a simpler basis for differentiation of the ECHO viruses.

Establishment of an etiologic relationship between the enteroviruses and disease is complicated by the fact that identical strains of virus are isolated from individuals with a wide variety of clinical diseases, as well as from healthy persons (45, 77, 93). The factors which control virus pathogenicity in relation to human disease are partly defined only for the polioviruses. The ability of the virus to produce paralytic disease in the monkey now serves as the only available criterion to differentiate human pathogenic and avirulent strains. As poor as this may be for poliomyelitis, similar characteristics are even less well defined for members of the Coxsackie and ECHO groups. Under natural conditions extrinsic factors which may either enhance or inhibit the invading pathogen are concurrent infection with other viruses and existing bacterial flora. Intrinsic participants are the mysterious influences of "host resistance." Elucidation of the role of these components may explain why some tissue systems will support virus multiplication without excessive cellular damage while, in other tissues, propagation of the virus occurs at the expense of the host cell.

Virus strains, other than poliovirus, have been encountered in which the clinical, epidemiologic, and serologic evidence point to their specific etiologic involvement in poliomyelitislike disease. These strains include Coxsackie A7 (38, 71), B5, ECHO 2, and ECHO 9 (90). It has been suggested that there is an increase in the severity of disease when multiple infections occur (21) although, in most instances, isolation and serologic data have failed to substantiate this idea. Since all populations are in a constant state

of shifting microbial parasitism, "interference" may play a significant part in preventing or reducing the severity of infection (41, 73, 80). The general subject of multiple infection will need further clarification before the phenomena of interference or enhancement in the production of disease is fully appreciated. In this respect the bacterial flora of the gastrointestinal tract, too, may be worthy of more attention than it has received.

RESPIRATORY DISEASE VIRUSES

In contrast to the enterovirus group, the present knowledge of those agents associated with the respiratory tract is better organized. However, there are many areas of interest where information is incomplete, and a vast area in which it is completely absent. Since the advent of antibiotics, respiratory diseases of nonbacterial origin have been recognized as a major public health problem. Much time and effort have been devoted to the isolation of etiologic agents. The agents which have been isolated and studied, however, account for but a small proportion of respiratory illness. Except in epidemics, the percentage of cases of respiratory disease of known cause remains small. Causal association is not easily established, since the mere isolation of an agent during the course of illness is not sufficient to establish that agent as having played a particular role in the disease syndrome. Many of the viruses have been found in healthy persons and could be present during illness by chance. Sprunt (89) has suggested the following criteria required to establish an etiologic relationship. The virus must be present frequently in the course of the suspected illness; specific antibodies must develop regularly following the illness; and transmission of the virus to susceptible hosts must cause a similar disease picture. Mogabgab (60) suggests the need for demonstration of a protective effect of vaccines in volunteers inoculated artificially, as well as in individuals exposed to natural illness, since the isolation of an agent during the course of illness with subsequent increase in antibody titer implies viral multiplication but not disease. Many similar sets of criteria have been proposed for the establishment of the etiologic role of newer viruses, but satisfying these criteria is not always practicable. Production of a similar disease picture in other hosts is not always possible and human volunteers are usually not readily available. Fulfillment of these criteria may also be attended by considerable difficulty since newer agents sometimes require several passages in tissue culture or other systems before ample amounts are available for further characterization. How the agent may be altered during the course of these passages is frequently unknown, although with more thoroughly studied viruses evidence indicates that significant changes may occur (30, 94, 100). The occurrence of such changes may result in the development of a virus strain unsuitable for the demonstration of specific antibody or production of a similar disease picture.

A number of newer respiratory disease agents are grouped together in the recently defined myxovirus group (1). At the time this group was defined, only influenza A, B, and C, and mumps viruses were included as known causes of human illness. Other viruses in the group were Newcastle disease virus and the virus of fowl plague. By defining certain of the broad physical and chemical properties of the initial members of the myxoviruses, it has been possible to group together new viruses of similar character in a more orderly manner. Although the antigenic characteristics of members of this group are not included among these properties, it is of interest that viruses exhibiting these broad properties also have some interrelated antigenic components. Mumps virus is antigenically related to Newcastle disease virus (32), croup-associated virus (10, 11), and Sendai virus (23, 32). Sendai virus is, in turn, related to the hemadsorption viruses (12). Subsequent to the isolation of Sendai virus in Japan (51) it was inferred, from positive results in serologic surveys, that the virus was present in the United States (48) and England (32) even though no isolations had been made. Since that time, hemadsorption virus type 2 (12), as well as mumps virus (32), have been shown to possess antigenic characteristics in common with the Sendai virus, necessitating a review of this finding in an entirely different light. The problem of establishing a specific agent as a cause of illness on the basis of serologic studies becomes increasingly difficult as newly isolated viruses are studied.

Viruses with a sound etiologic relationship to major human respiratory diseases were unknown, with the exception of influenza and psittacosis, until the isolation of the agents (43, 75) now known as the adenoviruses (26). New knowledge in the adenovirus group has accumulated rapidly in areas such as the properties of the viruses, epidemiology, etiology, serology, and vaccine evaluation. A review of the adenoviruses and other new respiratory disease viruses has been excellently presented recently (47). In a relatively short period of time, information concerning this group of viruses has become general knowledge with the result that more fundamental studies on the nature of the viruses themselves may be explored. Recent studies with adenoviruses have provided basic information concerning such questions as the factors responsible for the cytopathic effect in tissue culture (62) and the mechanism for the detachment of infected cells from glass on which they are grown (74). In addition, studies have illuminated the reproductive cycles (34) and the intracellular localization of adenoviruses in tissue culture (4, 24, 40). Techniques which have been applied for study of this group of viruses will have further extension in the characterization of newly recognized agents.

It was hoped that with the discovery of the adenoviruses, the etiology of much of the undiagnosed respiratory illness would be established. This, however, was not to be the case. It has been estimated in several studies (2, 27, 50, 69) either by isolation or by serologic techniques, that the incidence of adenovirus infection in civilian populations accounts for but a small proportion of the total respiratory illness. The situation in military recruit populations has been quite the reverse. In these populations, where a high incidence of adenovirus infection is observed, vaccination is successful in the preven-

tion of a large percentage of respiratory illness. Use of adenovirus vaccines in adult civilian populations is not indicated but the effectiveness of an adenovirus vaccine in children remains to be explored (28, 42).

An important trend in virology is the increasing tendency to group newer virus isolations according to similarities rather than to separate them according to differences. This has been well illustrated in the establishment of the adenovirus and myxovirus groups. Development of classification schemes is a sometimes thankless task, far removed from the interests of investigative studies, but one of the most helpful contributions directed toward a more systematic approach in a field which is extremely complex. Such contributions enable the individual investigator who isolates a new virus to subject it to basic tests for provisional classification, at least, before publication, The croup-associated virus (11) and the hemadsorption viruses (12) have been placed in the myxovirus group because they possess the basic characteristics required for inclusion in the group, but not all virus isolates are as easily classified. Studies with Price's JH virus, Mogabgab's 2060 virus, and the Eaton primary atypical pneumonia virus are beset with difficulties of propagation and measurement of serologic activity which make characterization more complicated (47). The properties of the respiratory syncytial viruses (13) do not permit their inclusion in any well-defined group at present. In many cases, inadequate laboratory tools are the cause of trouble and complete characterization of newer agents must await the development of improved basic techniques.

Two additional respiratory virus entities have been isolated recently and await proper classification. The first of these comes from the studies of Philipson & Wesslen (66) who isolated 19 agents from children suffering from nondiphtheritic croup. On the basis of serologic tests, the viruses appeared similar and were given the designation of "U" or Uppsala virus (63). Although apparently unrelated to croup-associated virus or to other previously isolated respiratory viruses, it is significant that the virus subsequently was associated with epidemic respiratory infection in adults (64) as well as in children. The illness in adults is milder and resembles common cold and is accompanied by a rise in neutralizing antibody titer of short duration. This infection was experimentally transmitted to volunteers who developed mild respiratory disease, a significant increase in hemagglutination-inhibiting and neutralizing antibody, and yielded virus on isolation attempts (65).

The second entity was isolated by Lennette et al. (52) from cases of pharyngitis and common cold. Four isolations were considered to be of the same virus type and designated "Coe" virus. No serologic evidence could be obtained to relate this virus to any of those recognized to date. A fourfold increase in neutralizing and complement-fixing antibody titers was observed in three patients from whom paired sera were available. In addition, serologic tests on 23 paired sera from patients experiencing respiratory disease at approximately the same time as the patients from whom "Coe" virus was

isolated, showed 22 per cent with a significant increase in titer. None of the patients showed serologic evidence of infection with adenovirus or influenza virus. On the basis of these serologic results these investigators postulated that the incidence of infection by this virus is greater than successful isolation attempts would indicate.

Further investigation is necessary to determine the role of the newer agents in the etiology of respiratory disease in various areas of the world. Such information may not be rapidly obtained or evaluated in proper perspective since the epidemiology of newer viruses is not well understood and isolation and identification of recognized viruses may be hampered by such factors as immune populations, or progressive antigenic variations of viruses in various hosts.

ARTHROPOD-BORNE ANIMAL VIRUSES

The wide biological attributes of viruses which are transmitted by arthropod vectors, touching broadly as they do upon the borders of many fields including zoology, entomology, and ecology, have invited a compelling interest in this diverse family of viruses.

That arthropods, particularly mosquitoes and ticks, are essential participants in virus maintenance and dissemination was first demonstrated for yellow fever, later for dengue, and more recently for several encephalitogenic viruses. Most recent studies have uncovered a considerable number and variety of such agents, widely distributed around the world, with different vertebrate host-vector associations accounting for different basic infection chains and epidemiologic patterns.

The tremendous rate of development in this field is attested to by the fact that only 20 years ago a popular textbook of microbiology (61) mentioned fewer than ten viral agents known or believed to be vector-borne. Ten years ago about 25 such viruses were referred to, although not in organized fashion, in the first edition of the most widely read textbook on viral infections of man (72). The current third edition of this book contains nine chapters describing about 50 of these viruses, now designated as the "arthropod-borne" animal viruses (73a). The contraction of this appellation to "arbor" viruses has been proposed but not universally accepted.

Although a little over 50 of these viruses have been more or less thoroughly studied, there is now a total of considerably over 100 such agents (7) and continuing search by workers in various parts of the world is swelling the list at an enormous rate. In many instances, however, information regarding their characteristics, geographic distribution, infection cycle, and pathogenicity is fragmentary and the problems of classification of such agents are greatly magnified.

Since biologic classification of this family of viruses is of limited value at present, the grouping system used is based on immunologic relations using the hemagglutination-inhibition, complement fixation, or neutralization tests. The hemagglutination-inhibition test provides, in some groups, the

broadest spectrum of antigenic overlap among these viruses, while the neutralization test has been the most specific. Much credit is due to Casals for improving a hitherto rather cumbersome and unreliable hemagglutination-inhibition test (8). Employing this technique as well as the complement-fixation and neutralization tests, he has been successful in creating some order out of chaos. Thus, arthropod-borne animal viruses have been divided into three serologic groups designated A, B, and C (9, 15). Additional study has made it possible to classify further the more closely related viruses of group A into allied subgroups (73a). Other remaining viruses are at present classed as "ungrouped." The results of recent studies with the ungrouped viruses indicate that additional serologic groups exist (7).

Group A includes the eastern, western, and Venezuelan encephalitis viruses and the Mayaro and Uruma (79) viruses, all isolated in the Americas. In addition there are Chikungunya, Middleburg, Semliki Forest, and Sindbis viruses from Africa, as well as two viruses recently isolated from mosquitoes in Malaya (5). Sindbis and western viruses are so closely related as to

make one subgroup; the remainder compose a second subgroup.

Group B lists such important viruses as yellow fever, dengue, St. Louis, Japanese B. Murray Valley, and Russian spring-summer encephalitis. A recent addition to Group B is the "bat salivary gland" virus isolated in Texas (6) and also in California (49) from Mexican free-tail bats. The significance of this virus is not yet clear. This large group is of interest also because its subgroups are more sharply defined. One subgroup is composed of St. Louis, Japanese B, Murray Valley, and West Nile. The second is named the "Russian tick-borne complex" (68) which includes the louping ill virus of Scotland; Russian spring-summer (Russian tick-borne) encephalitis, Biundulant meningoencephalitis (diphasic milk fever) and Omsk hemorrhagic fever, which are distributed in the Soviet Union from the Far East to Europe, and the Central European tick-borne encephalitis extending from Sweden and Finland in the north into the Balkan peninsula in the south. These viruses have been observed mainly on the Urasian continent, the major portion of which, because of its biologic and geographic unity, has been named the Palearctic zoogeographical area. Evidence that agents belonging to the Russian tick-borne complex occur outside this area has been presented by the isolation of a serologically related virus from monkeys and humans involved in an outbreak of febrile illness with fatal outcome in the Kyasanur Forest in India (99). Moreover, this virus complex appears to extend to the Oriental zoogeographical region since such an agent was also isolated in Malaya (83). Because distribution of viruses often appears to be directly related to the effort expended in attempted isolation in various regions of the world, it is probable that members of this virus complex are even more widely far-flung. It may be predicted, therefore, that agents related to the Russian tick-borne complex will eventually be demonstrated to exist also in the Western Hemisphere.

As an example of the complexities of the problem, there are now in Africa

alone at least 19 arthropod-borne viruses known to be pathogenic for man (84). Twelve of these have been isolated in the Union of South Africa³ since 1951; two so recently that they have not as yet been categorized (86).

The importance of a newly isolated virus is difficult to assess at first. When West Nile virus was isolated from an African woman with febrile illness in a remote region of Uganda (87) it appeared to have little significance as a human pathogen. Fourteen years later when it was encountered again it was shown to be responsible for epidemics in Israel (3, 35, 36) and the cause of disease, especially in children, in Egypt (59, 95). More recently it has been isolated in India (98) as was predicted on the basis of earlier serologic surveys (88). Similarly, Bunyamwera virus now has been shown to be more widely seeded in Africa than was at first realized, and with changing agricultural procedures, conservation methods, and transportation systems, it seems likely that Rift Valley. Bwamba, and Sandfly fever viruses may increase their range via the increased movement of their vertebrate and invertebrate hosts (86).

The immunologic overlaps among the arthropod-borne viruses offer the best taxonomic tools currently available but they have also raised important questions regarding diagnosis and epidemiology. Positive serologic reaction with an antigen does not always ascertain whether this was caused by homologous virus or by a heterologous response resulting from experience with a related virus (9, 78). Moreover, previous experience with one virus may so condition or orient the antibody response mechanism that subsequent infection with a virus of an allied antigenic composition will result in a level of antibody against the heterologous virus equal to or higher than the homologous virus (97). This phenomenon observed among the influenza viruses several years ago has prompted the scriptural pun "the doctrine of original antigenic sin" (22). Following an apparently universal pattern, the rickettsiae behave similarly and another excellent illustration of this situation has just been demonstrated in a rickettsial serologic study utilizing the more refined fluorescent antibody technique (37). Thus, the results of diagnostic tests and serologic surveys must be interpreted with caution until improved methods permit more specific results.

On the other hand, the broad serologic responses following inoculation of animals with vaccines of one type of virus, and the resultant cross immunity to challenge with other viruses of the group (39, 81), lends credence to the possibility of the preparation of a vaccine composed of one or two viruses which will provide effective cross immunity against many of the other viruses of the antigenically related group (53, 67, 70).

While the specific serologic reaction and the nature of its antibody "recall" is of considerable value in the characterization of the arthropod-

³ This is undoubtedly because there is located in Johannesburg a Rockefeller Foundation Insect-Borne Viral Disease Research Laboratory, working in collaboration with several local laboratories actively engaged in varied research.

borne viruses, it is obvious that other characteristics of importance should be explored for possible use in the more precise classification of these viruses. Physical and chemical properties added to immunological relationships are far more useful criteria for virus classification than are those of pathogenicity and tissue affinity. The absurdities which can arise from the utilization of disease-producing proclivities of a virus become readily apparent with only a casual review of the antigenic and pathogenic characteristics of members of the Russian tick-borne complex. These viruses are so closely related serologically that their separation as different virus entities is impossible. However, the diseases which they produce vary from louping ill, a severe disease of sheep but a mild influenzalike infection of man; to Russian spring-summer encephalitis, a severe encephalitis in man; to Omsk hemorrhagic fever which is without true central nervous system involvement but presents rather a generalized systemic hemorrhagic reaction. In sharp contrast, we have the poliovirus group or influenza group, each of which has types so far apart antigenically, that were it not for the identical clinical picture produced they could be considered wholly unrelated.

In the search for physical and chemical properties which would serve as more precise criteria for the classification of arthropod-borne viruses, several interesting areas have been uncovered. Limited studies on size determinations show the agents to be generally spherical and within the range of 15 to 40 mμ in diameter, although some may deviate to 70 to 122 mμ (85). The arthropod-borne viruses are inactivated by concentrations of sodium deoxycholate which do not apparently affect members of the enterovirus group or other related viruses (96). This distinction by bile solubility is reminiscent of a similar older application in the differentiation of pneumococci from related organisms. The sensitivity of these groups of viruses to contact with ether may also serve to distinguish them from enteroviruses. However, some of the respiratory viral agents also exhibit bile and ether sensitivity. Another interesting observation along these lines is the ready inactivation of the B group of viruses by the proteolytic enzymes trypsin, chymotrypsin, and papain, while the proteases have but little effect on members of the A group (14).

It is evident that there are insufficient data of a physical and chemical nature for the purpose of classification. Nomenclature, therefore, still depends largely on the isolation of the virus in suckling mice or tissue cultures, demonstration of its arthropod transmission, its pathogenicity and host range, its geographic distribution, and its serologic relation to other known viruses. This is a sizeable job requiring co-operating teams of scientists from various disciplines as well as sufficient funds for these elaborate procedures. It is still far from the capacity of the routine diagnostic laboratory.

CONCLUSION

Some of the implications of the recognition of so many new viruses in so short a time are at once obvious; the full impact will be more gradually revealed. Certainly, more viruses will be found and more gaps in the etiologic distinction of infectious diseases will be filled. Additional viruses will add complications to the existing difficulties of nomenclature and classification. Diagnostic procedures and serologic surveys will become more involved and evaluation of the results will require more judicious interpretations. However, the continued improvement of techniques and more precise methods will obviate these difficulties and eventually delineate the role of the multitude of viruses in health and disease as well as their position in the biologic order.

There is ample reason to believe that neither the recent viruses nor their diseases are actually new but only our recognition of them is new. New, however, are the opportunities to examine more closely the behavior of the viruses within and without their host cells. New, also, may be the approaches resulting from such observations to the understanding and ultimate control of the complex gyrations of these viruses through human and animal populations.

Bacteriologists, still searching for pathogens in man and animal by timehonored methods, would do well to emulate the modern virologist; for it would be surprising, indeed, if such effort did not lead to the development of new culture systems and the discovery of "new" bacteria and bacterial diseases.

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IMMUNOLOGIC TOLERANCE1

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INTRODUCTION

Immunologic tolerance will be considered broadly as any impressed diminution in immunologic responses below those which normally occur after known antigenic excitation. The antigenic stimuli may consist of treatment with cellular or soluble antigens, by exposure to skin grafting, or by environmental or induced infections.

It is becoming increasingly evident that the immunologic apparatus can give rise upon stimulus to two types of response, namely, delayed-type hypersensitivity (dermal, ocular, systemic) and the formation of serum-borne antibodies. In the area of immunologic tolerance, both types of immunological response must be considered separately, although the first of these may not come into play at times. For example, delayed-type hypersensitivity may not be encountered when soluble protein antigens are injected by the intravenous or intraperitoneal routes. The development of this form of immunologic response may require injection into the skin or the use of special techniques (e.g., mycobacteria as adjuvant, deferment of systemic antigenic stimulus by coating the antigen with specific antibody, or whole-body x-irradiation), and the time for its observation can terminate with the appearance of antibody in the serum.

Diminution of normal immunologic responses may be specific or nonspecific in origin. Nonspecific depression of the immunological apparatus can be secured in variable degree when cortisone acetate, heavy doses of wholebody x-irradiation (137, 139) or trypan blue (blockage of the reticuloendothelial system) are used prior to effective exposure to a primary antigenic stimulus. Such agents may interfere basically with the synthesis of antibody but leave operative the mechanism which is responsible for delayed-type hypersensitivity (112), or both sorts of response may be found suppressed. Also, certain diseases lead to nonspecific effects on the immunologic apparatus. The nonspecific anergy that often accompanies the onset of sarcoidosis, of Hodgkins's disease, and of lymphogranuloma venereum in the human being (116) tends to suppress the then-existing sensitivities of the delayed type, although the formation of antibodies may persist. Hypogammaglobulinemia, the congenital form in particular, causes a profound shift in immunological response, although delayed-type hypersensitivity can be found or excited in the absence of antibody-formation (58). In individuals with these diseases, deliberate attempts to induce delayed-type hyeprsensitivities are successful for variable periods of time, but the newly induced hypersensitivities are apt to wane within a few months (73).

¹ The survey of the literature pertaining to this review was concluded in April, 1959.

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Induced specific depressions in the functioning of the immunological apparatus will be given major consideration. In this area, some thought must be given to the relative stressing of the apparatus. Particularly with cellular materials, it will be seen that specific depressions may be secured with greater ease and with longer duration when the new antigens offered to the recipient do not furnish a "strong" antigenic stimulus. Either by reason of sheer numbers of antigens or by more chemical dissimilarity, some materials possess a greater degree of "antigenic obtrusiveness" than others.

In order to allow for all possibilities, Medawar (93) has postulated that suppressions may be the result of "central" impairment (to the apparatus itself), or conceivably may reflect "afferent" interferences (inactivation of antigen or its segregation and withholding from antibody-forming sites), or "efferent" blockage (protection of the target antigen from formed antibody). The scope of a role which can be ascribed to "afferent" and "efferent"

difficulties, obviously will be limited.

HISTORICAL

One of the first examples of unexpected immunologic inertness was encountered by Wells & Obsorne in 1911 (145, 146, 147). Young guinea pigs supplied by a particular breeder were being sensitized by corn protein (zein) for the study of anaphylactic shock. It was found that animals in later shipments were refractory to sensitization with this protein, a fact that was traced to a change in diet: the breeder had begun to supply "green corn, ears and all" to his breeding stock. Young stock could be rendered resistant at will to anaphylactic sensitization with zein by feeding moistened corn meal daily for six weeks as the chief food. Study showed that the feeding of oat protein rendered animals refractory to sensitization also with this protein. An initial period of hypersensitivity appeared to precede the refractory state.

In 1929, Sulzberger (134, 135) published his experiences in sensitizing certain stocks of guinea pigs to neoarsphenamine. An intradermal injection of 150 µg, would effectively sensitize with the production of delayed-type hypersensitivity (probably, however, a mixed type of reaction with participation of antibody), but no such response occurred if a prior intravenous injection of the same chemical (6 mg.) had been given. The significance of the refractory state was not appreciated generally, both because neoarsphenamine is an erratic sensitizer and because no general principle appeared to emerge.

A quite different instance of immunologic inertness was provided much later when studies were being made on the then-surprising finding that pneumococcal polysaccharide (considered a "hapten") was antigenic. Mice which received $0.5~\mu g$. of polysaccharide developed specific antibodies and resisted the injection of live pneumococci of the same type. The use of larger doses (0.5 mg. to 5.0 mg.), however, failed to immunize, and Felton & Ottinger (53) recognized this as a specific and durable impairment termed "immunological paralysis" (53 to 56).

In 1946, the writer (31) found that guinea pigs could be rendered tolerant to the sensitizing properties of chemical allergens of types other than arsphenamine by means of feeding these chemicals before sensitizing the animals with respect to the development of delayed-type, contact hypersensitivity. This work was undertaken for quite another reason than Sulzberger's and, indeed, required a technique other than intravenous injection; nonetheless, in principle it must be construed as a direct extension of Sulzberger's work. There was one important difference-the structures represented by chemical allegens (particularly picryl chloride and 2:4-dinitrochlorobenzene) were capable of being combined with proteins as allergenprotein conjugates; such synthetic antigens permitted studies on antibody formation. It was found that the tolerance was a type of full immunologic unresponsiveness (a name suggested to us by Dubos), since animals prefed with picryl chloride not only failed to develop delayed-type hypersensitivity to the simple chemical but resisted the development of anaphylactic sensitization even when injected with certain picrylated proteins.

Another finding that has proved to be of particular importance for the examination of the separate but associated problem of delayed-type hypersensitivity had by now become established, namely, cellular transfer. It was reported (30, 84) that white cells removed from appropriately sensitized guinea pigs would transfer to normal guinea pigs both delayed-type contact hypersensitivity to chemical allergens and the "core," at least, of true tuberculin hypersensitivity. [Extension of this work to the successful transfer of microbial sensitivities in human beings was being conducted convincingly

by Lawrence (85)].

Also, there was increasing attention to the role of white cells in the synthesis of antibody (gamma globulin) owing to the work of Fagraeus on plasma cells, while the experiments of Ehrich and Harris and White and Dougherty veered between implicating lymphocytes and phagocytic cells present in the reticulum of lymph nodes (89, 90). In any event, it was known that antibody could arise in normal animals following the transfer of separated and washed cells of stimulated lymph nodes from donor animals of the same species (33, 65), paralleling but distinguishably different from the cellular transfer of dermal hypersensitivity.

A different segment of the story appeared in an unexpected quarter. Owen, whose attention had been directed to red cell antigens of species hybrids reported in previous work by Irwin, Stormont, Cole, and Ferguson, studied the erythrocytes of dizygotic cattle twins, which almost invariably exchange blood early in embryonic life through anastomosis of the placental blood vessels. By painstaking immunological analysis, it was found in the majority of these cases that fraternal twins possess a mixture of two distinct types of erythrocytes corresponding to their individual genotypes. Owen concluded (102, 103, 104) that red cell chimeras had been established by fetal exchange of "embryonal cells ancestral to the erythrocytes of the adult animal," and persisted fully even after immunological maturity had occurred.

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Such was the contemporary setting, in immunology and biology, when Burnet & Fenner (28) postulated a mechanism for explaining the inertness of an animal vis-à-vis its own proteins and effete tissues. The reason might be the development in embryonic life of specific adaptive enzymes that would serve to degrade individuality "markers" without cellular disorganization and so allow the animal's own discarded tissues to go unrecognized as antigens by its immunological apparatus (whose functioning was related by the authors to a further change in these adaptive enzymes). Such enzymes were pictured as being capable of formation only before the set pattern of differentiation occurred during ontogeny, and persisting throughout life in descendents of the original cells. The mechanism pictured should work as well to degrade the individuality "markers" of other materials introduced into the plastic embryo. The points were made that,

if in embryonic life expendable cells from a genetically distinct race are implanted and established, no antibody response should develop against the foreign cell antigen when the animal takes on independent existence . . . and following a generalized non-fatal infection . . . of the embryo in utero, the animal after birth would be incapable of responding with antibody production to injection or infection with the same microorganism.

Burnet attempted unsuccessfully to demonstrate such tolerance, both with virus and with human erythrocytes. No direct evidence for the existence of such degradative enzymes has been produced as yet, and the challenging principle underlying tolerance remains unknown.

The hypothesis as offered served as a stimulus to many experiments, although obviously without it the biological milieu was fully prepared for exploration. Medawar, whose long-established concern with the problems of skin grafting had prepared him in a special way, turned to these newer developments with the aid of gifted colleagues. By 1951 and 1952, they were able to report (7, 23) that fraternal cattle twins (which exhibit a tolerant sharing of each other's red cell types) could accept skin transplants from their co-twins—even many sets in succession—but would reject selectively homografts from other full siblings. Turning to the conceptual basis of this tolerance which is established during embryonic life, Billingham, Brent & Medawar in 1953 (16) showed that long-term vascular anastomosis was not required in order to establish the tolerant state; living cell suspensions, introduced once into the bodies of embryo mice of a different inbred strain, would induce a tolerance such that skin transplants of the donor strain would be accepted specifically at any time after birth. As was to be anticipated on technical grounds, the durability of test grafts varied widely, from a few weeks to potentially lifetime, yet an eminently workable system was at hand.

From this classic experiment, the subjects of skin transplantation, organ transplantation, tumor transplantation, and infection with nonspecies-adapted viral agents, and prolongation of hypersensitivity reactions by transferred cells have by now grown into a special literature. The various aspects have been brilliantly reviewed in detail in recent publications (18, 25,

81, 87, 93, 94, 103), current progress is disseminated speedily in the "Transplantation Bulletin," and frequent conferences on homotransplantation are held (1, 2, 3). The present article will deal, consequently, with a consideration of principles rather than an attempt to present the factual material fully or in developmental order.

Another phase opened with the finding that lethal doses of x-radiation may be turned aside by injecting spleen cells and bone marrow taken from nonirradiated animals of the same or a closely-related species. Such studies have shown that the sparing occurs through repopulation of the injured animal by the donated cells, with "tolerance" assured by radiation injury to the recipient's immunological apparatus. The study of "radiation chimeras," hastened by the instant realization of radiation hazards, has developed principles that merge fully with aspects of tolerance induced by living cells placed in the embryo or the neonatal animal (2, 140).

UNRESPONSIVENESS TO STIMULI THAT EVOKE DELAYED-TYPE HYPERSENSITIVITY

Prior to further consideration of the problem of tolerance, it is necessary to recognize in what large measure our evidence has come from the blockage of homograft-rejection. Reasons for considering this to be the result of hypersensitivity (probably largely of the "delayed" type) are presented first. The great bulk of antigens which are present in cell surfaces and in cell constituents, giving rise to common antibodies and representing the "species" markers, do not come into play when transfers are made among members of a species. The important antigens are the more subtle ones that are designated as "individual-specific" because they reflect the genotype of the individual and differentiate him from his fellows.

Transplantation-hypersensitivity.—As a result of transferring skin from one genetically nonidentical member of the same species to another ("homografting"), there ensues an early vascularization and then, with the passage of about 8 to 12 days, a terminal rejection with necrosis and slough. The period oftentimes corresponds to the usual one for primary excitation of the immunological apparatus, and there is reason to view this as a reaction of delayed-type hypersensitivity with participation of lymphocytic cells (123).

On the other hand, repeated grafting with skin of the same origin causes a prompt reaction (92): the graft is not vascularized, but there is a cellular accumulation confined to the graft bed and the deeper layers of the graft's corium, and rejection is accomplished in less than six days. This event, also, may be viewed as a more prompt rejection owing to the previously established hypersensitivity of delayed- or tuberculin-type. It is more difficult to view it as a secondary stimulation of the immunological apparatus with an outpouring of excess antibodies. When, indeed, antibodies in adequate concentration are available, the second-set graft almost overnight turns into a "white graft" and there is little cellular accumulation. The writer agrees with Stetson (128) that the use of the term "transplantation immunity" to

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describe the usual type of rejection of "second-set" grafts is unfortunate; indeed, the word "immunity" in this connection being most ambiguous, we propose to use instead the term "transplantation-hypersensitivity." This points to the relation which exists between particular donor and recipient pairs, and it remains applicable whether either or both of the two major types of response of the immunological apparatus are involved in homograft rejection. It may be remarked that the Medawar group, also, has come to place in juxtaposition "immunity" (their previous term), and "sensitivity" (19). There are several reasons for viewing the rejection of grafts as being primarily a hypersensitivity reaction of the delayed-type; the available evidence has been spelled out by Lawrence (86, 87) At the same time, it would be presumptuous to believe that, when circulating antibodies do arise, they can play no part at all in homograft rejection. Stetson & Demopoulos (128, 129) have even found a few pools of rabbit immune serum which cause prompt rejection of skin that has been grafted to the ear of rabbits, and Voisin & Maurer (141) are inclined to view homograft rejection in guinea pigs as involving circulating antibody. Nevertheless, if we attempt to assess what role can be attributed to the two facets of the immunological response, it seems that delayed-type hypersensitivity can be established before the capacity to synthesize gamma globulins is developed, and that homograft rejection can occur very early in life, indeed (115). It was formerly argued that homografts are accepted and retained by hypogammaglobulinemic individuals because they make no antibody, and that antibody, consequently, must be the cause of graft rejection. It now appears (58, 59) that such individuals have a profound disturbance of their whole immunological apparatus, even though delayed-type hypersensitivities can, at times, be imposed directly by exposure to chemical allergen or indirectly by cellular transfer (59). The major share in graft rejection does not appear to be attributable to circulating antibody. Although antibodies soon arise, specific antibodies prepared in another animal are but rarely able to disturb skin homograftswhether mature, very young, or placed subsequently. Also, mechanical barriers which keep the recipient's own cells away from the homograft serve to prolong its survival (5), and so does implantation in the anterior chamber of the eye.

Another argument has been adduced by the writer. If graft rejection is a reaction of delayed-type hypersensitivity, then it would follow that individuals who do not readily undergo sensitization of the delayed type would be the most apt to retain homografts and to conceal the relative strength of their histocompatibility antigens. This may well be the reason that genetically unselected hamsters tolerate homografts to a surprising degree. It has even been stated that skin transplants are accepted between Syrian and Chinese hamsters (4). Billingham & Hildemann (22) found that within "closed" colonies sustained solely by pen inbreeding, hamsters accepted skin homografts for 100 days and more but rejected skin grafts much more typically when grafting was done between members of different colonies. Perhaps the

finding of Saenz (111) that 37 out of 44 hamsters failed to form granulomatous reactions when they were injected with attenuated bovine tubercle bacilli (BCG), may explain the sluggish responses by hamsters to grafts, since granulomatous responses and the production of a delayed type allergy often appear to be associated. If the relative capacity to give a granulomatous response were genetically controlled, the various observations would fall into line.

While the introduction into an animal of living tissue cells of another genetic constitution may be presumed able to give rise to delayed-type hypersensitivity (just as invading mircobial agents provoke "microbial" or "bacterial" allergy), it would be expected that histocompatibility antigens, if isolated, would require special application: they should meet with the same fixed limitations in inciting delayed-type hypersensitivity as are found with the use of other allergenic stimuli. The effective routes would be (a) intradermal; (b) parenteral if mixed with Freund's adjuvants (paraffin oil with mycobacterial cells); (c) intradermal (possibly), if present as an antigenantibody complex; or (b) and (c) combined. The attempts made to isolate histocompatibility antigens, described below, do indeed indicate that delayed-type hypersensitivity is provoked by special extracts of the revelant cells.

Chemical allergens.—Unresponsiveness may be established in mature guinea pigs vis-à-vis chemical allergens that give rise to delayed-type hypersensitivity when they are injected into the skin. Like tuberculin in tuberculin-sensitive individuals, the first of these, arsphenamine, requires intracutaneous injection to demonstrate delayed-type hypersensitivity. The other chemical allergens can be employed in the same way but, being fat-soluble, they also allow the demonstration of sensitivity quite delicately by contact tests made with solutions in triglyceride fats. The intravenous injection of neosalvarsan (neoarsphenamine), whown by Sulzberger (134) to render adult guinea pigs unresponsive to a later sensitizing procedure, has not been studied further because of peculiar and basic difficulties, perhaps genetic, in effecting sensitization with this chemical (135). The writer, in unpublished experiments, has confirmed the findings by substituting arspheamine for neosalvarsan: the number of sensitizing stimuli that are resisted vary substantially, but some animals remain unresponsive to many successive challenges. With other chemical allergens (e.g., 2:4-dinitrochlorobenzene, 1:2:4-trinitrochlorobenzene (picryl chloride), o-chlorobenzoyl chloride, and so on) unresponsiveness can be induced quite regularly by feeding (31, 37) prior to attempted sensitization, and inconstantly, e.g., with picryl chloride, even by one or a few contact applications to the skin. As will be mentioned later, very little of the amount fed enters the tissue as nonhydrolyzed 2:4dinitrophenyl-or picryl- groupings, but it is sufficient to change the status of the animals. Unresponsiveness is specific for the particular chemical, persists without detected diminution at least for 13 months after cessation of feedings, and is not upset by periodic attempts made to induce sensitization. The

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animals accept donated immune serum and react to it normally, without any concomitant change in inertness to delayed-type hypersensitivity. Further, they may be rendered contact-sensitive by the transfer of white cells (lymph node, splenic, exudative, and buffy coat) taken from sensitized donors (34, 84). This latter experience is often followed by a return to the unresponsive state (35). To explain the fact that specific unresponsiveness can be imposed on mature animals so readily, and endure for months, it has been suggested (37) that the addition of antigenically-determinant prosthetic groups to living tissues can effectuate a sort of long-lived "chimera," persisting because the altered tissue is the host's own.

Induced histocompatibility.—Erythrocyte chimerism, as shown by Owen in cattle twins and described above, has been detected in some pairs of dizygotic sheep twins (133) and in one human female, a twin (52). Vascular anastomosis between human fraternal twins is probably exceedingly rare.

Deliberate introduction of cellular antigens into embryos has been made by injection and, in birds, by artificial parabiosis as well (vascular anastomosis via the approximated chorioallantois of embryonated eggs). By these means, erythrocyte chimerism has come about in rats through injecting embryonic cells into the veins of embryos (109), and in birds as a consequence of parabiosis (18, 68, 117). Erythrocyte chimerism is relatively easy to evaluate, but it can occur in parallel with more silent types of chimerism which may be of much greater importance. For it is not erythrocyte chimerism that is sought commonly, but specifically induced histocompatibility. Consequently, the success of such injections in altering the natural state is usually assessed by skin- or organ- grafting after birth, and by attempted antibody stimulation, measuring thereby the relative inertness of the immunological apparatus in both of its manifestations. The usual rejection of grafts of tissue (skin, organs, bone marrow) made from one individual to another-unless the second partner is an identical twin-has been noted above

The electrifying success of Medawar's group in establishing tolerance to skin homotransplantation by injections made into the embryo opened a new chapter in experimental biology (13, 15, 18, 20). Their work revealed that the critical time for inducing tolerance varies with the species, and lasts longer than was originally supposed: the tolerance-responsive phase persists in rats for some days after birth (149), while the newborn mouse and chicken are just about at the end of this phase, necessitating intravenous injection of cells in order to distribute the cells and avoid a lag period of even one or two days. Of newly hatched chicks, for example, more than two-fifths can be rendered tolerant to homografting by early intravenous injection of homologous blood. Neonatal mice are now rendered tolerant in satisfactory proportion by injection (via a facial vein) of splenic cells, which provide a more concentrated source of "transplantation antigen" than peripheral blood (14), or subcutaneously with tumor cells given at the base of the neck within 3 hr. after birth for the establishment of tolerance to tumors (8).

The underlying principle of tolerance, consequently, would hardly be the "adaptive enzyme" hypothesis of Burnet and Fenner, as limited to the early stages in ontogeny. But it has become doubtful whether all the benefits of complete chimerism can accrue by the injection of adult cells, because the white cells which bear the transplantation antigens are immunologically competent and can express their ability by interacting with the tissues of the newborn, in measure as the latter possesses "obtrusive antigens." The more prominent of these are termed "graft-versus-host" reactions, and are discussed below.

With regard to success in homografting, it appears that the induction of tolerance requires the entry of living nucleated cells, taken at random from the donor's spleen, liver, lymph nodes, or peripheral circulation, and so on; lyophilized or heated cells can not serve as inducers. Although there is excellent reason to regard antigens which are closely associated with nuclei as playing the major role (see below), isolated nuclei themselves have failed thus far to establish tolerance. Some degree of early chimerism of a sort intimate enough to suppress the host's immunological reactivity seems required both for initiation and for maintenance of the unresponsive state.

As will be developed further, the necessary condition of useful chimerism may rest upon the provision of a continuing release of "homotransplantation antigens" to the recipient, serving thereby to maintain the unresponsive

state.

Of the many examples of induced tolerance, only recent ones will be mentioned. It has been found possible to make neonatal rats tolerant by transplanting thin discs of adult rat skin to them, so that many will show a prolonged survival of second-set homografts placed eight weeks later (96). The choice of species is important for this demonstration; in other animals studied free cells must be injected intravenously. The finding explains—demonstrably here on the basis of specific tolerance—several older observations. First-set homografts that have been made in newborn birds and mammals have enjoyed unexpected longevity, and some have been sufficiently reproducible for investigational purposes, for example, the "Danforth preparation" (48).

In goldfish (69), Hildemann has shown definite differences in survival between first-set and second-set grafts of scales. The extent of transplantation-hypersensitivity may be lessened by minimizing the absorption of antigen from first-set scales: sequential grafting from the same donor is practiced, and transplanted scales are removed as soon as vascularized (2 days). As found in all other homograft work, the sensitizing antigens are supplied only by living scales. First-set grafts became established in normal fashion even when the recipient had been stimulated to produce specific antibody by prior intraperitoneal injections of the intended donor's blood. The same immunizing blood deposited subcutaneously, however, gave rise not only to circulating antibodies but to transplantation-hypersensitivity as well, so that scale grafts would not take. This provides another instance of the separate-

ness of the two immunological responses, and recalls the parallel differences found by Billingham, Brent & Mitchison (21) in rabbits when white blood cells from the intended donor were given intraperitoneally and intracutaneously, respectively.

In another poikilothermic vertebrate (bullfrog larvae) Hildemann & Hass (70) have reported preliminary results on the development of homotransplantation-hypersensitivity to skin grafts; further, they were able to establish tolerance now and then by means of tailbud grafts made between larvae 6 mm. in length; the degree of tolerance, however, was often not

individual-specific.

Quite recently, Paterson [(106), cf. (36)] has been able to obtain concrete evidence that the lesions appearing in the brain and cord of animals within 7 to 20 days after the parenteral introduction of an emulsion of brain, paraffin oil, and dead mycobacterial cells are indeed allergic in character. For this, he made use of the principles discovered and elaborated by Medawar's group. Donors were injected with brain emulsions, and 7 or 12 days later cells from their lymph nodes were administered intravenously to recipient rats which had already been made tolerant to homologous cells. The recipients, thus rendered immunologically incompetent to reject the donated cells, developed encephalomyelitis with the same specific cellular lesions in the brain as are seen in active sensitization. It should be added that Paterson (107) has been able to make rats tolerant to antigens in rat brain by means of neonatal injection, so that rats do not experience allergic encephalomyelitis when injected during the first two weeks of life with brain substance emulsified with paraffin oil and mycobacteria.

Tumor transplantation.—Various attempts have been made to effect tumor transplantation from one strain of mice to another. Koprowski et al. (83) succeeded in establishing tolerance to a C₃H ascites tumor in a noninbred strain of mice (ICR) by introducing C₂H whole blood into fetuses; later, instead of challenging the injected animals with tumor as before at six to eight weeks of life, he established tolerance directly by intrafetal injection of the challenging ascites tumor itself, and indeed the latter grew spontaneously in the neck region two to three weeks after birth. Other tumors (DBA/2 lymphoma and P-288 ascites tumor) were passed similarly to ICR mice as a new host. But a new, secondary event, not seen with skin homografts, ensued: by polyploidy and "adaptation," these ascites tumor cells transformed gradually and became able to invade and to kill normal adult ICR mice as the new host. Through intensive effort, even two rat ascites tumors (AH 130 and Yoshida ascites) were adapted similarly to growth in normal adult ICR mice (82, 83), an unusual feat in view of some previous efforts to cross the species line.

By the same plan, Aust et al. (8) passed mammary carcinoma successfully to resistant strains of mice which had been rendered tolerant by neonatal injection within 3 hr. of birth.

Meanwhile, successful attempts had been made to prolong the period of

tolerance in turkeys so that the filterable Rous chicken sarcoma could "take" in mature and refractory turkeys. For this purpose, chicken blood cells injected into turkey embryos or turkey poults not older than 3 days sufficed (63, 117, 136). It seemed at first that the mechanism was the "Medawar" principle of unresponsiveness. Later work showed, however, that individual-specific cells were not required. Human Group A erythrocytes, formolized sheep red cells, and cell-free extracts of chicken cells all proved effective (64). It is probable that a heterophile antigen of polysaccharide nature is the active material, inhibition being perhaps analogous to Felton's "immunological paralysis" discussed later.

Speculation has it that perhaps Southam's observed rejection of cultured human cancer cells by healthy human adults, but acceptance as "takes" by cancerous patients, reflects rejection versus tolerance and the play of some such mechanism as we are discussing here (124, 125). More facts must be awaited.

Graft-versus-host.—When attempts are made to establish chimerism between embryonic tissue and immunologically mature adult spleen cells or peripheral leukocytes, it has been observed that the adult cells can be stimulated by antigens of the immunologically inert embryo and come to react against the latter owing to the development of delayed-type hypersensitivity and sometimes of circulating antibody as well. The stimulus is referable to antigens of the recipient's tissues which are not shared by the donor; the less "obtrusive" the antigen or groups of antigens the more minimal the effect. The first such instance, fowl spleen cells put into 18-day chick embryos, was noted by Simonsen (119): a severe hemolytic anemia appeared, accompanied by extreme splenomegaly owing to colonization of the organ by the mature cells, and death usually occurred within 2 weeks after hatching. In one critical test with new-hatched chicks, Cock & Simonsen (41) showed that the F₁ offspring of two diverse strains of inbred chickens will be attacked by cells of one parent but that adult F1 cells will not harm young individuals of the parental strain. Using relative splenomegaly as an index of reaction, Simonsen et al. (120) have studied similar situations occurring between five strains of inbred mice and their F1 offspring. In only two combinations were the F1 offspring unaffected by adult cells of either parental strain. These findings are supported by the independent observations of Billingham. From their analysis, Simonsen and his colleagues have concluded that "tolerance is more solidly acquired to transplantation antigens that are determined by recessive genes than to those determined by dominant genes."

Billingham & Brent in 1957 had reported the first instance of a graftversus-host reaction in the mouse, which varied in intensity from a lethal effect within 2 to 3 weeks [(14) cf. (140)] to a stunting ("runt disease"), when adult spleen cells were injected into newborn mice in particular combinations of genetically stabilized mouse strains. Close examination revealed, however, that even apparently successful cellular chimeras (adequate for

full tolerance of homografts) might show evidence of interaction as abnormalities or involution of lymphoid tissues (12). Billingham was able to induce runt disease with peripheral leukocytes, and pointed to its possible importance in the etiology of certain types of hemolytic disease occurring in human babies.

Most likely a direct counterpart is provided by instances of radiation damage in mice, in which repopulations with other, even some heterologous, cells become possible (2, 45, 100). In these, it has been noted that donated spleen cells have interacted to produce a fibrotic, wasting disease with atrophy of lymphoid tissue. As Trentin pointed out in 1956 [cf. (140)], this is an instance of graft-versus-host, not vice versa, since skin homografts or

heterografts are being well tolerated at the time of late death.

Reversal of graft tolerance.—Spontaneous loss of tolerance occurs whenever the host becomes able to react to the "individual-specific" antigens of the graft. In a chicken that had been an embryonic parabiont, it was observed by Billingham, Brent & Medawar (18) that spontaneous loss of tolerance affected synchronously both the previous state of erythrocyte chimerism and the tolerated skin graft. Likewise, Medawar & Russell (95) found transplanted adrenals and skin homografts of the same source to be rejected together, late in the life of rather tolerant rats. The time of spontaneous rejection varies widely, every degree of tolerance being encountered. Brent (25) has observed that incomplete tolerance in mice is seen as a "low-grade reaction resulting in epithelial weakness and ulceration, progressive contracture, swelling and scabbing-culminating in the total breakdown either a few or many days" beyond the mean survival time. But the fact that these same mice tolerated second-set grafts longer than would be expected after destruction of the first set led him to conclude, ". . . once tolerance had been induced the animals probably never returned to a state of normal immunological reactivity. Tolerance may therefore be regarded as a permanent impairment of immunological response."

Much study has been given to the genes which control the relative "strength" of the histocompatibility antigens towards other mice, the most important usually proving to be those located at the H-2 locus on the

chromosome (123).

Of greater interest are the manipulative techniques which have been found to cause, at will, a slough of grafts dwelling in stabilized tolerance. Since the host's own immunological apparatus has become unable to respond to the antigens which emanate from the graft (the "signal not being picked up," as it were), the impasse can be broken by introducing normal lymphoid tissue of the host's genetic constitution. Such tissue recognizes the antigens as foreign, and slowly develops a state of hypersensitivity towards them; the graft is approached and infiltrated by round cells and a "first-set" type of rejection ensues. A "second-set" type of rejection will be effected, however, if the transferred cells have been allowed to acquire their hypersensitive status beforehand. For this, an animal of the host's strain is allowed to reject a skin homograft which is genetically like the tolerated graft, and its

stimulated lymphoid tissue is used in the transfer. The first of these tools was introduced by Billingham, Brent & Medawar in 1954, the second (which we rephrase here as "adoptively acquired hypersensitivity"), by Mitchison in 1953 in connection with the rejection of lymphosarcoma (98, 99); it was then applied by Billingham et al. to skin homograft sensitivity [(15) cf. (141)]. The latter procedure has been employed frequently, both to test graft identity versus adaptation of the graft to its new host (81), and as a method for studying acquisition by lymphoid tissues of the capacity to function in homograft-rejection. In the mouse, which is endowed with a high metabolic rate, only the lymph nodes on the side draining the skin homograft acquire much stimulus to react against tissues living in stabilized tolerance, and the optimal time for harvesting is about 5 to 10 days (99, 101). The requirement for avoiding contralateral nodes does not hold to such an extent in the guinea pig (26) or the rat (96), The lymphoid cells are ineffective if frozen and thawed, or heated, and exhibit little activity if injected subcutaneously in the living state.

A recent application of the procedure of "adoptively acquired hypersensitivity" is provided in the transplantation of adrenal cortical tissue between adult mice of genetically different lines. Tolerance can be established by neonatal injection of spleen cells corresponding in source to the intended adrenal tissue, and checked by skin homografting (95). Transplanted cortical tissue vascularizes and becomes functional, for adrenalectomized bearers of such grafts survive without needing elevated salt intake. The transfer of activated lymphoid cells, however, causes rejection of the functional tissue ("immunological adrenalectomy"), and homografted skin is sloughed at the same time. Likewise, in heavily irradiated mice which have accepted and are subsisting on foreign bone marrow, rejection of the new marrow can be secured by injecting "unirradiated lymphoid tissue of host type" and the mice die in consequence (140).

Mitchison & Dube (101) studied the relative roles of delayed-type hypersensitivity and serum antibodies in causing the rejection of Sarcoma I implanted in the mouse [cf. (71)]. Cellular transfer on the sixth day was effective in establishing homograft-hypersensitivity, before the transferred cells had led to the appearance of antibody. The two events are separable, but Gorer (60) has contended that both components are perhaps needed

for homograft rejection.

Mention should be made that Stoerk (131) found a special labile humoral factor in the serum of rats in which solid tumors were actively regressing, which proved to possess activity and stability after separation from other serum proteins. Gorer (60) has shown a positive correlation, using ascites tumors and mouse leukemia, between the times of incipient homograft rejection and appearance of agglutinins which react with red blood cells of the donor mice (6). Others believe that red cells sometimes share some one antigen in common with fixed nucleated white cells, and that in such cases hemagglutination would be expected.

Antibody-induced unresponsiveness.- In some instances, the presence of

antibody in the circulation alters fundamentally the way in which donated cells are accepted. A novel example is provided by the findings of Harris et al. (66, 67). When rabbit lymph node cells, stimulated by bacterial antigens in vitro, are transferred to other rabbits, bacterial agglutinins appear in the serum of the recipients. But if a recipient rabbit has experienced injection of normal lymph node cells from the intended donor some days prior to the transfer of the latter's stimulated lymph node cells, the output of antibody is found to be markedly depressed. The "rejection" is specific for the lymph cells of the particular donor individual, and depends upon the development of antibody in response to the individual-specific antigens of lymphoid cells, as Harris says. It seems possible that rejection of the donated cells may rest not along on the acquisition of circulating antibody with respect to the donated cells, but also on a concomitant development of hypersensitivity of delayed type towards these cells [cf. (92)]. Even with the use of lymphoid cells of pen-inbred rats with reduced genetic heterozygosity, Stoerk et al. (132) were able to establish a temporary resistance to "takes" of Murphy rat lymphosarcoma. A fundamental question appears to be whether circulating antibodies are directed against the actual histocompatibility antigens or against cytoplasmic or surface antigens of cells. Kidd, for example, could suppress growth of Brown-Pearce tumor cells in rabbits with a rabbit antibody prepared by injection of cell-free tumor extracts (80), and hence probably devoid of histocompatibility antigens.

In another sense, we may point to a quite different principle that has impressed us in studies on delayed-type hypersensitivities to chemical allergens in guinea pigs. With single allergens applied as stimulus to the skin, both delayed-type hypersensitivity and (later) antibody appear. Instances have been noted in which, if a stimulus is provided selectively so that antibody production is incited first, it is difficult to evoke delayed-type hypersensitivity later. The principle might explain the protection from allergic encephalomyelitis that follows at times when injections of brain substance without adjuvant are given first (43). The impetus for development of delayed-type hypersensitivity—the mycobacterial cell with paraffin oil—is offered with the brain substance only later. Perhaps a similar mechanism is at play when allergic encephalomyelitis is diverted by a sort of mechanical "afferent inhibition" by removing the regional lymph node

draining the injection depot (44).

Another instance which perhaps may rest on this principle is the finding of Billingham & Sparrow (24) that dissociated rabbit epidermal cells, if given by the intravenous route to a rabbit, will prepare it to tolerate a later homograft from the same rabbit for a period two to three times as long as a normal "first-set" homograft can survive. The principle appears to rest on prior antibody response to histocompatibility antigens, for only living epidermal cells or whole blood of the same individual were able to establish this form of tolerance.

Another example of antibody-induced unresponsiveness is afforded by the

"enhancement" phenomenon of Casey, elaborated into a basic laboratory tool by Snell (60, 75, 123). By treating the intended recipients of certain tumors for some days in advance of transplantation with generous amounts of lyophilized, dead tumor tissue (or normal tissues from the same or partly related strains of mouse), antibodies arise and alter the recipient's behavior. After hemagglutinating antibody is found, implantation of the tumor is followed by a remarkable enhancement of tumor growth, seen particularly with tumors which possess a low invasiveness. The precise mechanism is not settled. Snell believes it likely that the effective homograft antigens bind with antibody and are "walled off" from the lymph nodes, a development of homograft hypersensitivity being slowed thereby. This would be one of the few instances of "afferent inhibition," defined earlier. Billingham, Brent & Medawar, who have corroborated the finding with skin transplants, hold a similar view. Kaliss (75), however, points to substantial difficulties in accepting the above hypothesis and argues that serum must act directly on the graft, perhaps by allowing its adaptation to the host. Tumors, once started, are known to brush aside minor immunological insults and to maintain growth, even when a simultaneously borne skin homograft of the same genetic origin is sloughing. Search for the responsible antigens of the tissue that set in motion the enhancement phenomenon has been started by Kandutsch et al. (76, 77); the antigen seems to be a protein-carbohydrate complex, perhaps a mucoprotein.

Individual-specific antigens.—Whereas only intact living cells seem to be capable of establishing individual-specific tolerance, recent work discloses that their antigens can be separated by special methods and used to induce homograft-hypersensitivity. Except for the evidence of great lability and the mode of assay, the search scarcely differs in motive from similar quests for individual-specific tissue antigens which are being pursued in other laboratories. Kandutsch & Reinert-Wenck (77) have examined tumors for the individual-specific antigens which engender antibodies and enhance growth of the tumor. The enhancing antigens were present in all tissue fractions examined, but the activity was not associated with RNA or DNA proteins, or with lipide, and withstood contact with RNase or DNase. The presence of an essential protein was inferred from lability to heat, alcohol (80 per cent) phenol (90 per cent), and trypsin. There was strong evidence for a polysaccharide also; sodium periodate (M/100) had a markedly destructive effect. This oxidizing agent splits carbon-carbon bonds vicinal to hydroxyl or hydroxyl and amino groups and degrades carbohydrates, including the human blood group substances and other polysaccharide-containing antigens. Billingham, Brent & Medawar (19), continuing their studies on the isolation of "homotransplantation antigens," have prepared new types of antigenic cell-free extracts from spleen, thymus, and lymph nodes by special methods which avoid the use of Ca++ and Mg++, for these ions cause the active material to coprecipitate with the inactive DNA proteins. Their new material behaves much like the Kandutsch preparation from mouse tumor. It is free

of DNA protein, and is thought to represent a rather unstable lipoid-protein complex with determinant groups which are resistant to a large battery of enzymes but which are degraded by a crude preparation of *Trichomonas foetus* enzyme and by a weak concentration of potassium periodate. In the latter respect, the material would behave like human blood group substances, although it is considerably more labile. Whatever the structure, the activity of the preparation is unique among known cellular antigens, for a dose of 2.5 to 8.0 mg., prepared from an intended donor-strain of inbred mice and injected into the intended recipient of a skin homograft, will sensitize the latter (but for a short period of time only) so that it will reject skin of the donor strain.

A newer approach to the detection of "homotransplantation antigens" has arisen, based upon the developing concept that delayed-type hypersensitivity determines homograft-rejection. Hypersensitivity responses are being studied in albino guinea pigs, since this species is one in which delayed-type hypersensitivities come to excellent expression and in which the principles of cell transfer are well established. First, guinea pigs that had rejected skin homografts were studied to learn whether delayed-type hypersensitivity actually had been evoked (26, 27). It was found that they were indeed hypersensitive to cell-free extracts prepared from the tissues of the skin donor, as revealed by a tuberculinlike response upon intracutaneous injection; neither serum proteins of the donor nor similar extracts made from the spleens of control guinea pigs provoked any similar reaction. These sensitizations appeared regularly in all recipients upon graft rejection, but they varied considerably in intensity. Besides cell-free extracts, lymphoid cells of the same origin could be used to make the skin tests although due allowance had to be made for nonspecific toxicity.

Viewed the other way about, the hypersensitive state of the recipient guinea pig toward such antigens should reflect changes induced in its lymphoid cells. When these were harvested and injected intracutaneously into the skin donor in the viable state (a requirement) a positive, slowly developing reaction ensued; either the supply of "test antigen" provided by the milieu of the skin was higher, or the trapped cells remained undispersed (97, 142). When the last-described experiment was carried over to rabbits, particularly strong dermal reactions were noted, possibly denoting production of antibody by locally injected cells (34, 110). As could be anticipated, however, the phenomenon is not reproduced in mice, since mice do not exhibit cutaneous hypersensitivity of the delayed type.

Positive intracutaneous tests made in this way are stated to measure the antigenic potency of extracts with a need for only one-tenth the amount of material that is required to sensitize mice and induce accelerated rejection of skin grafts (26).

Makinodan et al. (91) have begun to define the antigens of rat tissue which render mice hypersensitive to grafts of rat bone marrow. From perfused liver, the antigens found in microsomal and mitochondrial fractions

were resistant to nucleases and proteases, while their nuclear fraction resembled the behavior of Billingham et al's former preparation.

The tissue polysaccharide of Harris & Simons (64), having an apparent heterophile activity, has been mentioned previously.

IMMUNOLOGIC UNRESPONSIVENESS INDUCED BY COMMON ANTIGENS

Unlike homograft tolerance, in which only the duration of graft acceptance provides a clue to the existence of partial or intergrade effects (rejection being occasioned when tolerance is lost to certain ones, not necessarily all, of the transplantation antigens) an animal's relative ability to synthesize antibody can be subjected to quantitation. As previously stated, the latter function is an immunologic response which is dissociable from responses of delayed-type hypersensitivity (the closest analogy to homograft rejection). In the area of erythrocytes and soluble proteins, delayed-type hypersensitivity usually does not come into play, and the issue is reduced to an examination of antibody response. Since it has been shown that antigen tolerance is sufficiently durable with the use of "non-surviving antigens," as Owen has phrased it, special studies have become possible. In these, just as in tolerance to homografting considered above, there is increasing evidence that the mechanism is based upon retention of the antigen, or of its essential configurations, intracellularly. In contrast to homografts, there is no self-renewed antigen supply and commonly the tolerance dwindles eventually [e.g. (122)]. Indeed, the most durable degree of unresponsiveness is usually established by materials which are resistant to the animal's metabolic processes. As with homografts, tolerance is initiated by administering the antigen while avoiding excitation of the antibody-producing mechanism.

We should mention here a type of "natural experiment" that has been inferred from studies in the Rh system; its mechanism would be of unknown nature. There is evidence that Rh-negative female fetuses, when carried by Rh-positive mothers, may acquire some sort of experience with the Rh antigen that persists in influencing their immunological apparatus through the years of sexual maturity (105): more than half who marry Rh-positive husbands fail to produce circulating Rh antibody during 3 pregnancies, as contrasted to one-quarter of those who have been born to Rh-negative mothers (103). The apparent durability would be suggestive of some type of hidden chimerism. No similar differential has yet been observed, however, in the incidence of erythroblastosis occurring in the offspring of the two groups.

Polysacchardides, chemical allergens.—Certain materials appear to provoke, even in adult animals, a specific depression of antibody synthesis. These include pneumococcal and Friedlaender polysaccharides on the one hand, and also simple chemical allergens which are so introduced as to avoid any immediately effective stimulus of the antibody-producing cells.

When pneumococcal capsular polysaccharides first became available and

were found capable of immunizing mice, it was noted that-unlike whole vaccine—there was an unusual dose-response effect: the smaller doses immunized against virulent pneumococci, the larger ones failed to do so [(113, 114); cf. (108)]. By 1942 Felton & Ottinger (53) had found that mice receiving the larger doses (e.g., 0.5 mg, intraperitoneally) gave no evidence of producing antibody even upon further stimulus with pneumococcal vaccine, that the unresponsiveness was essentially type-specific, and that it persisted at least for 15 to 18 months. Felton termed the state "immunological paralysis" (53 to 56). Much attention has been given to the phenomenon since. Kaplan, Coons & Deane (78) by means of fluorescent-antibody have visualized the initial widespread dissemination of Types II and III polysaccharide throughout the tissues of mice. By the end of one month, there is sufficient polysaccharide present to dispose of I131-labelled specific (rabbit) antibody by prompt elimination from the blood, and Dixon, Maurer & Weigle (50) pointed to prompt catabolism without evidence of fixation on tissues. Felton himself had found that polysaccharide-bearing mice required several multiples of the antibody needed by normal mice for passive protection. Felton had been able to recover type-specific polysaccharide by extracting the tissues of mice as long as one year later, and Stark (126) reported C14-labelled polysaccharide (Type I) remaining in the spleens of mice without loss in radiocarbon for one year. Alterations in the polysaccharide, however, commence earlier. Even with the use of 16 times as much Type III polysaccharide as is required to induce paralysis in mice, Kaplan et al. (78) found antibody-reactive polysaccharide present only scantily 6 months later, chiefly in the histiocytic and reticular cells of the spleen, the Kupffer cells, macrophages in the heart, stromal macrophages of the kidney medulla, and the walls of the peritubular plexuses in the kidney. Likewise upon direct extraction, specifically reactive polysaccharides appeared to dwindle in amount with time (54, 55, 56) and eventually could not be located. Stark (126, 127) studied the antigenicity of crude aqueous extracts of spleens taken from paralyzed mice; antigenicity decreased gradually, particularly between the third and the eighth weeks, until only about 2 per cent of its initial value was present twelve months later. Such loss could be the result of structural alteration in the polysaccharide, deacetylation or complexing with other constituents, or-as many have come to suppose-as a result of interaction with antibody.

The thought has been voiced repeatedly that "immunologic paralysis" is not what the name implies, but that antibody synthesis proceeds more or less normally. Antibody as produced would be taken up on polysaccharide depots and disposed of (50, 57, 78, 126, 127), so that a "dynamic situation" obtains. With this in mind, Stark (127) has studied the rate of decline in the antigenicity of aqueous splenic extracts of paralyzed mice versus successive administrations of specific immune serum. Although no significant decline in extractable antigen occurred spontaneously over 75 days in paralyzed control mice, the net effect of giving mice 9 injections of the same rabbit anti-

Type I serum during 70 days was, indeed, to cause a constantly accelerating loss in recoverable antigenic polysaccharide.

To this reviewer, it remains as a basic observation that the mice remain fully "paralyzed" for much more than a year, long after these various changes in the 'loading' have occurred, and he knows of no evidence which would indicate that antibody has been discovered within a paralyzed mouse. No information has been produced as to whether the polysaccharide residual a year later would suffice to take up administered antibody, or whether such antibody would abruptly cancel the 'paralyzed' state. Indeed, to judge from certain experiments with native proteins injected neonatally (vide infra), intracellular antigen and external antibody might be expected to coexist and not intereact. Would it not be equally likely that an initial general cellular loading is required to influence the small, sensitive areas which are to become actually 'paralyzed,' and that the excess polysaccharide is so large that, being able to absorb donated antibody, it can lead to misinterpretation of the true process? Further studies on unresponsiveness arising from pneumococcal polysaccharide may prove it to be analogous to other instances of tolerance to common antigens.

The possibly analogous instance of "immunologic paralysis" induced in turkeys by intraperitoneal injection of a Forssmanlike polysaccharidic material (8) was mentioned earlier; turkeys could be made tolerant to Rous chicken sarcoma by injecting them, newly hatched, with whole chicken erythrocytes, stromata, and cell-free extracts, or human group A cells (which possess Forssman antigen), but not with human Group O cells.

The unresponsiveness which can be induced by chemical allergens to the development of both delayed-type hypersensitivity and output of circulating antibody has been mentioned above. The example provided by intravenous injection of neoarsphenamine has not provided an opportunity to study circulating antibody; picryl chloride, on the other hand, has lent itself to such examination. When the compound is introduced orally into adult guinea pigs in 3 mg. doses daily until 50 mg. has been fed, there is a substantial

change in the immunological apparatus (37).

By the use of 1-C14-labelled picryl chloride, one may search for picryl groupings retained in the tissues. It is found that the relatively large dose given per os results in the excretion of large amounts of its decomposition product, picric acid, over the course of a week or so, and that the picryl groupings retained in the tissues are insufficient for definitive autoradiography even when the specific activity is 2.0 mc./mM (38). The change in the status of the animals is, accordingly, accomplished with only trace 'loading' of the tissues at best. In this circumstance, the animals do not produce antibody and they do not develop antibody or the anaphylactic statein contrast to their normal fellows-when serial injections of the chemical are made intracutaneously (32). Also, they do not remove antipicryl antibody when it is introduced intravenously (9). If one uses picrylated homologous proteins to sensitize instead of such intracutaneous injections of the

simple chemical, the animals are found to resist becoming anaphylactically sensitized, and they do not produce antibody possessing picryl specificity. But, as noted below, the resistance so expressed can be broken down by the use of a carrier protein which is antigenically more "obtrusive" (10, 37).

Soluble proteins.-Most antigens, in contrast to the special ones considered above, must be offered at a "null point" in the biological development of the capacity to synthesize antibody if unresponsiveness is to ensue. The introduction of mammalian proteins (bovine serum albumin) into young rabbits, if commenced just after birth and continued for some weeks or months, produces a clear-cut depression of the antibody-forming response, observed when the same protein is injected after a rest of four to eight months (39, 40, 49, 61, 62, 122, 143). In the earliest experiments, massive doses (as much as 40 to 50 gm.) were administered and persisting unresponsiveness was noted, even 7 months past the time when antigen remained in the blood (49, 74). For example, Cinader & Dubert (39, 40), using large amounts of human serum albumin, found even 600 days later that the rate of elimination of antigen from the blood stream was that of nonstimulated rabbits. Later on, when the effects of small doses (less durable) were under scrutiny, it was learned that even oral administration of bovine serum albumin (100 mg.) on the day of birth would ensure passage into the blood stream of rabbits and establish a specifically refractory condition in many of them (122). Here we find interpreted the 1911 observation of Wells and Osborne, namely, that young guinea pigs raised on corn protein would resist anaphylactic sensitization to this particular protein.

Various types of immunological stress applied to specifically refractory animals have yielded interesting information. For example, attempts made to overcome the refractory state appear to be more successful as the vigor of the antigenic stimulus increases. As noted above, Chase (32) found that guinea pigs made specifically unresponsive to picryl chloride would not become anaphylactic when picrylated guinea pig protein was the challenging antigen, but it proved possible to break through the unresponsiveness by injecting picrylated bovine gamma globulin (10). Smith & Bridges (122) found that injecting water-in-oil emulsions of bovine serum albumin into the skin of specifically unresponsive rabbits broke through the refractoriness more readily than did intravenous injection of the protein alone: it is possible, of course, that the route chosen resulted in preferential stimulation of nonsplenic sources of antibody production which had been less influenced by the original neonatal injection. In the "solid" resistance induced to human serum albumin, Cinader & Dubert (39, 40) were able to get the rabbits to recognize as antigen a chemically modified human serum albumin (coupled with diazotized p-amino benzene sulfonic acid), and antiserum produced in two of the rabbits contained a proportion of antibody molecules which reacted with native human serum albumin as well. By this indirect means, an effective stimulation had been provided. One of the two rabbits finally became capable of stimulation by native human serum albumin itself, although it is difficult to judge the role of protracted stimulations versus aging. Another significant observation was provided by Downe (51). When fetal and newborn rabbits were rendered unresponsive by injections of whole chicken serum, immunological stimulation with turkey serum gave rise to antibodies as expected. Nevertheless, the influence of the unresponsive state intruded, for the resulting antibodies were found to be devoid of the usual fraction that cross-reacts with chicken serum proteins.

Information offering some concept of the modus operandi has been provided by the thorough studies of Smith & Bridges [(122) 1958 et antel, who have made single injections of protein antigens into the peritoneal cavity of neonatal rabbits and challenged with two injections of antigen four months later. Substantial blockage was found by using 20 to 100 mg, of bovine serum albumin, whereas smaller quantities gave variable results, perhaps a "partial tolerance." Consonant with this, it was seen that proteins containing small amounts of contaminating antigens produced unresponsiveness to the major antigen only. To maintain the unresponsive state, periodic challenging injections of antigen were required; and without these, susceptibility returned within three to six months. The amount of bovine serum albumin required to support the tolerant state must apparently be greater than 1012 to 1013 molecules. It was also found that 'immune' cells borrowed from other rabbits (in connection with an injection of the antigen) would cause antibody to appear in the unresponsive rabbits equally as well as in normal ones (143), and that the rabbits were left still unresponsive after the transferred system was spent. Accordingly, there was no evidence that antibody was taken up on "antigenic depots" or that the presence of specific antibody would vitiate the mechanism (probably intracellular) which maintains unresponsiveness.

Most recently, Crampton et al. (47) have studied neonatal rabbits rendered unresponsive to iodinated protein (8 per cent iodine) by repeated injections made into the peritoneal cavity. The unresponsive rabbits handle I¹³¹-labelled iodoproteins quite in normal fashion, the label localizing in spleen and liver cytoplasmic granules. These workers offer a new hypothesis: they suggest that if an antigenic stimulus does cause nascent antibody to be synthesized by antibody-forming cells which contain such localized protein (or hapten), interaction intracellularly could result in a sort of selective cellular suicide. Gradual elimination of the "loaded" cells, occurring in this way or alternatively by dilution (cellular division), would explain recovery from the unresponsive state.

The unresponsiveness impressed by mammalian proteins upon newly hatched chicks or embryos is less durable than that in the rabbit, six weeks being about the limit of depression of antibody synthesis. Curiously, little attention appears to be directed to studies with purified avian proteins, for example, turkey serum albumin, for suppressive effects in the chick. With injections of bovine serum albumin, Wolfe et al. [(148) cf. (138)] found that both embryos and hatchlings exhibited a definite depression of response at six weeks but a decided waning in the weeks immediately following, as

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Stevens et al. (130) found with human serum albumin in 10- to 18-day embryos. The effect is proportional to the dose. Reinjected at the six-week period, the unresponsive animals cleared specific antigen from the peripheral circulation only slightly less rapidly than did normal control chicks in which antibody synthesis was evident (148); this feature, not observed with experimental rabbits, has not yet been examined closely. Hirata (72) found, however, that only 20 mg. of S³⁵-labelled bovine serum albumin was needed to establish unresponsiveness, that most was excreted within one week, and that tolerance persisted through six weeks when only traces of retained antigen were still present. These traces would hardly suffice to permit an hypothesis of antibody formation accompanied by absorption on antigenic depots; perhaps, indeed, antibody synthesis or antibody liberation is truly held in abeyance.

Even with such an artificial preparation of altered proteins as Old Tuberculin (Weybridge), Weiss (144) surprisingly found that a proportion of the guinea pigs which had been injected with it while in the fetal state appeared to resist attempted sensitization to tuberculin at eight weeks of age. The sensitizing administration consisted of OT itself or of living or chemically killed avirulent bovine mycobacteria (BCG). The observed tolerance did not persist long, for hypersensitivity appeared after a subsequent injection of living BCG.

By using the elegant method of chick-turkey embryonic parabiosis, Hašek (68) was able to induce in chicks a significant depression of response to turkey serum antigens and, indeed, there was a transient maintenance of red cell chimerism and production of turkey serum proteins.

Erythrocytes.—When erythrocytes are used, cells of precisely the same genetic origin must be available for later attempts to incite a production of antibody; particularly in view of the polyglot diversity of erythrocyte antigens found in most domestic birds, one may expect that certain individuals, possessing more "obstrusive" antigens, will prove to be less useful than others in the study of antigen tolerance.

Billingham, Brent & Medawar (17) injected chicken red cells (free of white cells) into chick embryos, and into chicks at hatching. Little antibody response resulted upon a later injection of the same cells given after hatching and growing. In the same way, Hašek found that chicks which had been embryonic parabionts would not make antibody to each other's cells, in adult life, but would respond to the cells of other chickens. Similar experiments with red cells of other species appear to be beset with more difficulty. With erythrocytes of aves, goose blood injected into embryo ducks partially suppressed a later antibody response, although nearly complete suppression lasting for 25 weeks could be established by repeated injections started in new-hatched ducklings (68). Many other intraspecies transfers failed, but one of the latter, turkey blood introduced into embryo chicks and vice versa, showed a significant and moderately durable suppression in Simonsen's hands (117). While the introduction of mammalian cells into chick embryos

gives a much less complete suppression—indeed, none may be found (11), it often seems to lengthen the time for initiating antibody synthesis [(118), cf. (103)]. Even in newborn rats and rabbits, the injection of mammalian blood may delay and depress the antibody response to a later injection (11, 103).

Bacterial cells.—If particulate complex antigens such as bacteria are given to embryos, later exposure usually uncovers only a diminished antibody response, although suppression is often found (8a, 29, 79). Smith & Bridges (121) found no suppression with several types of bacterial cells. Obviously, the initial distribution of such antigenic material will be less widespread than in the case of single molecules, partly in accord with the pattern of phagocytosis. It should be recalled that amounts of pneumococcal vaccine more than equivalent in polysaccharide to a 'paralyzing' dose failed to inhibit antibody production (114).

Depression has been noted also when injections are made in the very young, e.g., to Salmonella pullorum vaccine in chicks (29), to Trichomonas foetus in cattle (79) but failure was experienced in neonatal rabbits by Smith & Bridges (122), who note further an immune response occurring late in the neonatal period. Perhaps, in addition to the limitations of 'loading' the cells, the time of interference will remain critical; as noted above, the most sensitive time for influencing chick embryos is recorded as being between 17 and 19 days. The expected duration of tolerance must also be considered. The thought has been expressed that Cohn's (42) experiments, started on the fourteenth day of embryonic life with challenge deferred until the twelfth to fourteenth week after hatching, were destined for failure because the tolerant state had then passed away (80, 138).

CONCLUSION

It will be evident that immunologic tolerance represents a "central" impairment of antibody formation; only a few special instances can be explained by "afferent" means of keeping stimuli from reaching centers which are capable of response. As antigens with radioactive labels indicate (47, 126), there is usually a prompt and widespread entry into the microsomal fractions of cells. The extent to which overloading of the cells is necessary may perhaps be dictated by the amount of antigen which can delay stimulation of the immunological apparatus, until some situation, as yet unknown, has become established. It seems probable that exceedingly small amounts in sensitive areas can block the apparatus. By cellular division, the initial loading can diminish, requiring reloading; the need will be maximal for substances which can undergo metabolic degradation, minimal for materials (chemical prosthetic groups, certain polysaccharides) which resist it. Homografts appear to sustain the supply of antigenic material by continued vital function. What they contribute, however, may not be readily metabolized. Local retention of antigen is probably witnessed by the "recall flare" reaction described by Rapaport and Converse (1957): after placement of a second-set graft, the rejection site of the former graft bed flared at the time of reaction in the second-set graft. So far as analysis of "transplantation antigens" is known through their capacity to induce homograft-hypersensitivity, they are "individual-specific" and not "tissue-specific." Evidence implies that these are associated with something like mucoproteins, having both proteinic and polysaccharidic composition and labilities.

Under favorable circumstances, inhibitions of both delayed-type allergy and of antibody production have been established together. Seemingly, the former is the one which is concerned in homograft rejection.

Whether the laboratory models which have been erected apply to man is still to be learned. Inertness of cancerous individuals to human tissue culture cancerous cells is striking. Likewise, one can keep in mind the experiences of Longcope & Mackenzie (88) in immunization of persons with horse serum; there were a few individuals who did not synthesize antibody, in whom horse serum could be detected circulating for many weeks.

As the above account will reveal, there seems to be little merit in differentiating between "immunological paralysis," "immunological tolerance," and "acquired tolerance,"

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COMPLEX LOCI IN MICROORGANISMS1

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The scope of this article has been determined by the editors of the Annual Review of Microbiology who, in assigning the topic, requested that particular attention be paid to the arrangement of loci concerned with sequential steps in biosynthesis. Therefore, our review consists of two sections, one dealing with the structure of gene loci and the other with their chromosomal arrangement. While retaining the term "loci" in the title, we wish to point out that in our terminology "locus" is synonymous with "gene locus," and designates the genetic region occupied by a certain gene in any of its forms. The term "gene" we ordinarily apply in a more specific sense, as referring to a particular form occupying the locus. For example, by hisB locus we mean the region that may be occupied by any hisB gene, either wild type (hisB+) or mutant (e.g., hisB-14).

STRUCTURE OF GENETIC MATERIAL

Mendel's law postulates that the unit of heredity, the gene, may exist in two forms, one being dominant and the other recessive when both are present in the same organism. Early research in genetics soon revealed that the number of forms (alleles) of a gene may be greater than two. In fact, experiments with the "white" locus in *Drosophila* indicated that the number of alleles may be considerable, and also that different alleles originate by mutation. Thus, the existence of different variants of a gene has been recognized for a long time and constitutes one of the basic principles of genetical theory.

About 30 years ago Dubinin (47), Serebrovsky (155), and others investigated the phenotypes of a number of "achaete-scute" alleles in *Drosophila melanogaster* (each of which lacks specific individual bristles on head, thorax, or scutellum so that each allele produces its own characteristic bristle pattern). They found that the different phenotypes could be arranged in a definite series according to bristle patterns, and also that heterozygotes lacked only those bristles which were affected in common by both participating alleles. They concluded that the serial classification of alleles according to bristle patterns has its counterpart in a similar arrangement of parts of the achaete-scute locus. On this assumption they divided the locus into twelve elementary subunits, called "centres," each affecting a particular

¹ The survey of the literature pertaining to this review was concluded in April, 1959.

bristle or group of bristles. It was assumed that each allele arose by a change involving a certain combination of these centres. According to this theory, the achaete-scute locus is made up of separate, regularly spaced, and lineally arranged functional units [Dubinin (48, 49)].

These investigators regarded achaete and scute as parts of a single achaete-scute gene locus. As indicated above, their initial efforts were concentrated on analyzing its structure in terms of the bristle patterns produced by various alleles and those produced in heterozygotes combining two different alleles. In the course of the work, however, they obtained evidence that the two parts of this complex locus can be physically separated, and also that crossing over can take place between the achaete and scute subunits. Genetic analysis of the mutant sc8, which carries a long inversion in the X chromosome, showed that one of the breaks is in the achaete-scute region, just between achaete and scute. In other words, the scute portion of the locus has been transferred by the inversion to a position near the base of the X chromosome whereas the achaete portion remains near the tip [Patterson & Stone (137)]. Cytogenetic analysis revealed that the achaete-scute region is represented on a salivary gland chromosome map by two discs, one including bands 1B1-2 and the other bands 1B3-4. A cytological analysis of the sc⁸ inversion by A. A. Prokofieva-Belgovskaya showed that one of the breaks had occurred between bands 1B1-2 and 1B3-4 of the Bridges (14) salivary gland chromosome map and thus that 1B3-4, presumably representing the scute portion of the achaete-scute locus, had been transferred to a position near the base of the chromosome while 1B1-2, presumably representing the achaete portion, had remained in the original position [Serebrovsky (156)]. At about the same time, Dubinin and his collaborators (50) reported crossing over between achaete and scute, having found four crossovers among about 75,600 flies examined.

Unfortunately, this fine work, which had its center in Russia, was terminated by the suppression, under the influence of Lysenko, of genetical research. The results, however, were indicative of physical differentiation within a gene locus, and suggested that crossing over, as well as breaks resulting in chromosomal rearrangement, may occur within a locus. They also showed that in heterozygotes different alleles may complement each other, either completely or partially. A structural model which would account for their observations was outlined by Raffel & Muller (145) as follows: "... it is not far fetched to imagine that the 'gene for scute,' as recognized by the test of allelism of its mutations, may nevertheless consist of an undetermined number of parts, some of which may become separated from others by breakage without such separation resulting in the loss of the reproductive power of any of these parts. . . . These parts, then, might themselves be denoted 'genes,' and the whole a 'gene-complex,' or the parts might be called 'sub-genes' or something equivalent, and the whole a 'gene'...."

Shortly after the termination of these studies of the achaete-scute region

of the *Drosophila* X chromosome, work was begun on the closely related problem known as pseudoallelism. In 1940, Oliver (129) described the occurrence of crossing over between two alleles of the "lozenge" locus in *Drosophila*; and later Chovnick (22), Green (72, 73), Green & Green (74), Lewis (114 to 117), Welshons (177), and a number of others analyzed similar phenomena in other regions of *Drosophila* chromosomes. Results of the studies reveal a uniform pattern of behavior throughout: sites of alleles which give rise to phenotypically similar mutants can be separated by crossing over; and certain pairs of such alleles show complementation in heterozygotes, whereas others do not.

At present there is no general agreement with regard to the interpretation of these results. Some geneticists still hold the view that the gene, defined as an ultimate unit of recombination, corresponds to the unit of function and mutation. Others, who are ready to accept the findings of work with microorganisms as probably applying also to higher organisms, interpret pseudoallelism as evidence of the complex nature of the gene locus.

EVIDENCE OF COMPLEX LOCI

With the development of very sensitive techniques for detecting the results of recombination between different mutants, evidence began to accumulate—first in work with fungi and soon afterwards in studies of bacteria and bacterial viruses—about the occurrence of recombination between alleles of the same locus. Within a few years enough data were available to justify conceiving of a gene locus as composed of a large number of linearly arranged units (sites), alterable by mutation and capable of recombining with nonhomologous sites of a homologous locus.

Complex loci in fungi.—Among the first contributions of microbial research to the problem of complex loci was the discovery by Bonner (11) and by Giles (63) of recombination between alleles in Neurospora. The purpose of Bonner's experiments was "to determine whether... genic complexity could be found in Neurospora, and if so whether it could be demonstrated for a gene affecting a known reaction." His results were positive: he observed recombinants in crosses between five alleles of the "Q" locus, which block the ability of the organism to convert 3-hydroxyanthranilic acid to niacin Similar results were obtained by Giles in tests with several alleles of the inositol locus.

A considerable amount of information as to mutability patterns, nutritional requirements, and complementation, as well as recombination, has been accumulated with regard to alleles of several other loci in *Neurospora*. This is summarized in Table I.

The study of complex loci in Aspergillus nidulans developed from work by Roper (151) who found close linkage among three biotin mutants. His discovery was the result of a deliberate search for linkage between similar mutants, prompted by the working hypothesis [Pontecorvo (139)] that close linkage might be expected among some of the genes controlling any one

TABLE I

LIST OF GENE LOCI IN MICROORGANISMS FOR WHICH COMPLEX STRUCTURE HAS BEEN REPORTED

HAS BEEN REPORTED

KEY: *=probably two adjacent loci, although single bifunctional enzyme not excluded mut=different rates of back mutation demonstrated temp=temperature-sensitive allele demonstrated linkage =linkage relations with outside markers disturbed for one or more alleles but not for others leaky =some growth in absence of specific growth factor supplement for one or more, but not all, mutants of locus su =allele-specific suppressor demonstrated on pos = position effect on expression of adjacent or nearby genes dominance =one or more mutant alleles recessive while others semidominant or dominant to wild type growth =one or more alleles differ from others in allowing growth under special nutritional conditions nu =one or more alleles differ in nutritional requirement

Organism	Locus	Activity affected	Compl tation dem stra	units ion-	Single- site mutants analyzed	Minimum No. sites demon- strated	Heterogeneity among mutant sites	References
Phage T4	*II		2 -	A	102	45	mut, leaky,	(7, 9, 10, 52)
			2 В	83	28	temp		
	h		1		6	6	temp	(165)
1	h+		3		12	6		(51)
Phage P22	c		3				linkage	(112, 113)
Phage \(\lambda \)	c		3					(95, 96)
Salmonella	his E	?	4		13	13	mut, pos, linkage	(76, 78, 79)
	his F	See Fig. 2	1		37	34	mut, pos, linkage, growth	(78, 79)
	his-A, H*	See Fig. 2	2 -	A	25	25	mut, pos, linkage, growth	(78, 79)
			2 -	Н	2	2	pos, linkage	(78, 79)
	his B	See Fig. 2		4	34	31	mut, pos, linkage, growth leaky, temp	(78, 79)
	his C	See Fig. 2		1	35	33	mut, linkage, leaky, temp, su	(78, 79, 100, 159)
	his D	See Fig. 2		2	61	55	mut, leaky, linkage	(76, 78, 79, 79a)
	his G	. 3		1	11	11	mut, linkage	(76, 78, 79)
	try A	anthranilic acid			10	10	mut, su	(37, 38)
	try B	anthranilic acid to indolegly- cerol-phos- phate (i-g-p)			32		mut, su	(37, 38)
	try C	i-g-p to indole			11		mut, su	(37, 38)
4	try D	indole to tryptophan		1	12	12	mut, su	(37, 38)
	pro A	between		1	20	20	mut	(38)
	pro B	glutamic acid and glutamic γ-semialdehyde (g-s)		1	20	20	mut	Miyake (unpublished)
	С	between g-s and proline		1	17	17	mut, leaky, nu	[(38), Miyake (unpublished data)
	D	before glutamic acid		1	4	4	mut, growth	[(38), Miyake (unpublished data)

COMPLEX LOCI IN MICROORGANISMS

TABLE I (Continued)

Organism	Locus	Activity affected	Complemen- tation units demon- strated	Single- site mutants analyzed	Minimum No. sites demon- strated	Heterogeneity among mutant sites	References
	cys A*		1 1	6 2	6 2	mut	(29, 83)
	cys B			13	13	mut, nu	(23, 24, 34)
	cys C			8	8	mut	(23, 24, 30)
	cys D			8	8	mut	(23, 24, 30)
	cys F			6	3	mut	(23, 24, 30)
	thr A			5	5	mut	[(38), Glanville (unpublished data)]
	В			5	5	mut	[(38), Glanville (unpublished data)]
	C			2	2	mut	[(38), Glanville (unpublished data)
	isl A			9	9	mut	[(38), Glanville (unpublished data)
	ilva A			5	5	mut	[(38), Glanville (unpublished data)
	В			7	7	mut	[(38), Glanville (unpublished data)
	C			2	2	mut	[(38), Glanville (unpublished data)
	leu		4	11	11	mut	[(38), Margolin (unpublished data)
	ad B			2	2	mut	(39, 40, 42, 188, 189)
	C			2	2	mut	(39, 40, 42, 71, 188, 189)
	E		$2 \frac{E_1}{E_2}$	16	16	mut, su	(39, 40, 42, 71, 188, 189)
	ath A			8	8	mut, su, nu	(39, 40, 42, 188, 189)
	C			2	2	mut	(39, 40, 42, 188, 189)
	D			5	5	mut	(39, 40, 42, 188, 189)
E. coli	gal 1, 4	UDP-transferase	1	2	2		(97, 98, 102, 104, 126)
	lacı	galactoside- permease	1	3	2	su, mut	(25, 103a)
	id	tryptophan synthetase				leaky, mut, su, immunological, accumulations	(26, 108, 182, 184, 185, 187)
	str			2	2		[(38), Hashimoto (in manuscript)]
	su str			2	2		[(38), Hashimoto (in manuscript)
Neurospora	ad-3*	3	ad-3A	35			(43, 44, 45, 66)
			2 ad-3B	156		temp, leaky, mut, linkage	
	ad-4 (ad-F)	adenylo-succinase	7	123		mut, leaky, temp	(65, 68, 133, 180)
	ad-5	AICAR to FAICAR to IMP	3				(65)
	ad-E	IMP to AMPS	3	>20			(65, 133)
	am	glutamic dehydrogenase	2	4	4	leaky, mut, temp, linkage	(53, 54, 56, 57, 134, 135, 136)
	arg	arginosuccinase	1	4	4		(55, 128)

data)]

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TABLE I (Continued)

Organism	Locus	Activity affected	Complemen- tation units demon- strated	Single- site mutants analyzed	Minimum No. sites demon- strated	Heterogeneity among mutant sites	References
	arg	citrulline+ aspartate to arginosuccinate	1	5	1		(128)
	arg-5	3	1	19			(20)
	arg-1	?	4	40			(20)
	arg-10	?	2	13			(20)
	cys-1	7	1	22			[Parker, cited in (20)]
	cys-2	?	1	21			[Parker, cited in (20)]
	cys	sulfate to thiosulfate	2	2	2	growth	(138)
	albino-1 albino-2		2	2	2	linkage	(3, 6)
	his-1	imidazole- glycerol PO ₄ ester dehydrase	1	3	3		(1, 59)
		ester denydrase	2	13			(20)
	his-2	5	2	14			(20)
	his-3	L-histidinol	4	?			(65)
		dehydrogenase	1	15			(20)
	inos	3	2	15	3	temp, mut, su	(63, 64, 66, 67, 132, 138)
	iv-1	?	2	3	2		(138)
	lys-1	5	1	8			(20)
	lys-3	3	1	5			(20)
	lys-?	3	3-4	46			[Ahmad, cited in (20)]
	orn-2	,	2	40			(20)
	PABA	3	1	4	3		(60)
	pan-2	ketovaline to ketopantoate	5	37	11	mut, linkage	(17, 18, 19, 65)
	pdx	?	2	3	2	temp, growth	(122, 138, 160)
	pyr-3	?	2	3		temp, su	(124, 125)
	td	tryptophan synthetase	2	24		mut, temp, su, leaky, accumulations, immunological	(11, 12, 81, 103, 167-172, 176, 181, 182, 186)
Aspergillus	bi	3	1	3	3		[(140, 151); Roper, in (142, 143)]
	ad-8	3	1	8	7	leaky, su	[(144, 154); Pritchard cited is (141, 142)]
	ad-9	3	2	6	6		[(15, 16); Calef cited in (141) Martin Smith cited in (142)]
	pro-1, 2	3	pro-1 pro-2	-	2 2		[Forbes, cited in (141, 142)]
	ad-1, 3	?	ad-1	1	1		(140, 141, 142)
	1 ., .		2 ad-3	1	1		

O

Sac

Zy

TABLE I (Continued)

Organism	Locus	Activity affected	Complemen- tation units demon- strated	Single- site mutants analyzed	Minimum No. sites demon- strated	Heterogeneity among mutant sites	References
	acr-1	?	?	3	2	dominance	(153)
	5*	sulfate to sulfite	2 51	7	2		(94)
			s0	2	1		
Saccharo- myces	isoleucine	?	1	2	2	mut	(150)
	ad-1	?	1	4	4		(146, 147, 148)
	ad-2	5	1	4	4		(146, 147, 148)
	ad-3	3	1	9	6 or 8	leaky	(146, 147, 148, 149)
	ad-4	?	1	13	11	leaky	(146, 147, 148)
	ad-6	7	1	29	26	leaky, mut	(146, 147, 148)
	ad-5, 7	?	ad-5 2 ad-7 ad-5, 7	7 8 11	5 4 11	leaky	(146, 147, 148)
Zygo- saccharo- myces	ad-7	?	1	11	9	su	(110)
	ad-2	?	1	3	3		(110)

series of millimicromolar biochemical reactions, of the type observed in the synthesis of vitaminlike substances [McIlwain (118)]. Roper interpreted his results as demonstrating close linkage among three loci. In later experiments he obtained evidence that, although each of the mutant alleles was recessive to wild type, no two of them complemented each other: all heterozygous combinations of the alleles produced mutant phenotypes. This finding led Pontecorvo (140) and Roper (152) to propose, as an alternative to the interpretation assuming close linkage among three genes, a second and more plausible explanation, namely, that the three biotin mutants were alleles of a single gene locus, resulting from mutations at three different sites within it.

[(8

d in

141);

Recombination has been detected between alleles of several other loci in Aspergillus. The most extensively analyzed are two adenine series, ad-8 and ad-9. At the ad-8 locus four mutational sites, separable by crossing over and arranged in linear order, have been recognized (144), and at the ad-9 locus five sites have been established (15, 16, 119).

In the yeast Schizosaccharomyces pombe, Leupold (109, 110, 111) has analyzed recombination among 11 noncomplementary mutants of the ad-2 locus, and found evidence of nine sites. Recombination has also been found between two mating-type alleles. In another yeast, Saccharomyces, Roman (146, 148) has studied several alleles at each of seven adenine loci, with the results shown in Table I.

Complex loci in bacteria.—The first conclusive evidence of complex genic structure in bacteria proceeded from work done at the Cold Spring Harbor laboratory during 1953 and 1954. In order to study patterns of spontaneous and induced mutability in various mutant characters, we had accumulated

about 200 auxotrophs and about 50 galactose-negative mutants of Salmonella typhimurium, strains LT-2 and LT-7. The first step in the proposed study was to determine which among phenotypically similar mutants were allelic. This we did by means of the transduction technique developed by Zinder & Lederberg (190), working on the assumption that recombinants would not be detected in experiments involving two alleles, whereas they would appear in experiments combining two nonallelic markers. Although the method was successful, the basic assumption proved to be inaccurate. Our results show clearly that mutant alleles do recombine, although with a frequency considerably lower than that obtained in transduction experiments between a mutant and its wild type allele. They suggest that "a gene locus extends over a section of a chromosome, and that changes occurring in different regions of this section give rise to different alleles. They also indicate that regions within a section may separate, and recombine—by a process analogous to crossing over-with homologous regions within a locus of another chromosome." [Demerec et al. (41)].

Moreover, the work revealed that allelic recombination is a common phenomenon, for it was detected in one serine, one galactose, two proline, three adenine, three cystine, three tryptophan, and four histidine loci. These early findings received ample confirmation from the results of extensive experiments carried out subsequently by members of the Cold Spring Harbor group and reported in several papers (23, 24, 29, 30, 32 to 37, 39, 40, 76, 80, 83, 130, 188, 189). A large collection consisting of more than 1200 auxotrophs and sugar-fermentation mutants was used in these experiments. On the basis of two criteria, namely, linkage relations and positions of biosynthetic blocks, more than 60 gene loci were identified; and in more than 55 cases in which two or more alleles were available for study, either all the alleles or a large percentage of them were found to be nonidentical (i.e., recombination took place between them). This finding justified the conclusion that nonidentical allelism (complex structure of loci) is not a special feature of certain gene loci but a general property of all.

Complex loci in bacterial viruses.—By means of an ingenious technique he had developed, Benzer (7, 9) was able to detect recombination between rII mutants of bacteriophage T4 of Escherichia coli and to establish the complex structure of the rII locus. The technique is based on the observation that wild-type phage produces plaques on either of two bacterial hosts, B or K, whereas a mutant of the rII group produces plaques only on the B host. In a cross between two relatively stable rII mutants, any wild-type recombinants that arise, even in a proportion as low as 10⁻⁸, can be detected quantitatively by plating on K. With this technique Benzer analyzed a large number of rII mutants and showed that, although all are located within a sharply defined portion of the phage linkage map, recombination readily occurs among a large proportion of them. According to the criterion of complementation, the mutants fall into two groups, A and B—mutants of the A group complementing those of the B group but not one another, and vice

versa. Benzer also found that group-A and group-B mutational sites are located in different but adjacent regions on the linkage map of the rII section.

Results of similar analyses carried out with three other phage loci are given in Table I.

NATURE OF COMPLEX LOCI

Genetic constitution of a locus.—On the basis of data from biological experiments, we can visualize a locus as a segment of chromosome which comprises smaller sections (sites) separable from one another by recombination. A site, then, is a subunit of a locus, at which mutation may occur but within which recombination has not been observed and presumably does not occur. Identification of a site obviously depends upon the sensitivity of the method available for detecting recombinants: a method with high resolving power is required for analysis of the composition of a locus. It seems very likely that the methods developed for work with bacterial viruses [Benzer (7)] and with certain loci in bacteria [Demerec (30)] have absolute resolving power, that is, are sensitive enough to detect recombination between two markers if there is a possibility of its occurring.

Experiments with phages and with Salmonella typhimurium, in which the occurrence or nonoccurrence of recombination serves for the identification of sites, have detected three types of alleles in the same locus: (a) those which recombine with one another to produce the wild type; (b) those which recombine to produce wild type with certain alleles of type (a) but not with all; and (c) those which do not recombine among themselves but do recombine with all other type (a) mutant alleles. Type (a) alleles are the result of mutations at different sites of a locus; they have been called nonidentical alleles by Demerec (31) and heteroalleles by Roman (146). Type (b) are presumably caused by changes affecting two or more adjacent sites (multisite mutations); and type (c), called identical alleles by Demerec and homoalleles by Roman, result from repeated (but not necessarily identical) changes at the same site.

The known properties of multisite mutant genes, briefly summarized, are as follows: They fail to recombine with a number of closely linked markers. As a rule they are found at any one locus less frequently than are single-site mutants. They occupy a well-defined region of chromosome. Auxotrophs produced by multisite mutations do not revert to prototrophy. In a case where it was possible to study crossing over between a multisite mutant marker (hisB-22 of S. typhimurium) and adjacent markers, the crossover frequency was lower than that expected from the results of other tests involving the same single-site markers (76). These observations indicate that multisite mutations are complex changes, and suggest that they may be chromosomal aberrations (inversions or deficiencies).

If certain assumptions are made, one can estimate the number of sites per locus by several different methods. One of these methods bases the calculation on the total number of mutant alleles found and the number of identical alleles among them. A similar principle was applied by Muller (127) in making one of several estimates of the number of genes present in D. melanogaster. The basic assumptions are: (a) mutations at different sites occur with equal frequencies, and (b) every mutational event is expressed as a mutant. Frequently mutating sites ["hot spots" of Benzer (7)] are present in material obtained by spontaneous mutation (7, 9, 78). Furthermore, there may be many sites at which a mutation does not lead to a detectably altered (mutant) phenotype. This calculation thus provides only an approximate estimate of the minimum number of sites.

Roman (146) used a formula suggested by J. Crow: n = N/I where n is the number of sites, N the number of allelic combinations tested, and I the number of combinations producing no recombinants. His estimates of minimum numbers of sites in five adenine loci of Saccharomyces are as follows: ad-3, 36; ad-4, 39; ad-5, 77; ad-6, 135; and ad-7, 64. A parallel estimation for seven histidine loci in Salmonella yielded the following values (78): hisE, 196; hisF, 456; hisA, 676; hisB, 385; hisC, 613; hisD, 620; and hisG, 121.

An estimate by this method of the minimum number of sites in the A portion of the rII locus can be based on the data about spontaneous mutability at that locus given in Figure 2 of Benzer & Freese (10), if one omits the values for two sites which mutate very frequently. The estimated number is 65. A similar estimate for locus rIIB is not feasible, however, since apparently a large proportion of its sites mutate with higher than average frequency.

Another method of reaching a minimal estimate of the number of sites in a locus is comparable to an earlier calculation of the total number of genes in *D. melanogaster* (127). The estimate is based on the ratio between the total map length and the length of the smallest recombination fraction measured. Pontecorvo (142) followed this method in calculating minimum values for three loci in *Drosophila* (bx, 37; lz, 187; w, 70), two in *Aspergillus* (bi, 1000; ad8, 1800), three in phage (rIIA, 330; rIIB, 269; h, 153), and two in *Schizosaccharomyces* (ad2, 65; ad7, 251). The method assumes that all the sites tested will recombine, per unit distance, as freely as they have been observed to recombine with other, more distant, markers. This assumption neglects considerations of interference and the effects of specific mutational changes on recombination frequencies.

In several organisms, numerous nonidentical alleles, presumably at independent sites, have been identified (Table I). Thus, it is evident that the number of sites comprising a locus can be large and, if the estimates are indicative, may considerably exceed 100. We may reasonably assume that loci differ from one another with respect to the number of sites.

Order of sites.—There are several ways of determining the order of sites within a locus and thus constructing a genetic map of the locus. The simplest but least reliable way is to arrange the sites according to the frequency with which the markers recombine, on the assumption that frequency of recombination is directly correlated with distance between markers. Since it is known that a number of factors may influence recombination frequency (76, 78),

this method reveals the order of sites only approximately, but it has proved to be of great value [see (8, 9, 110)].

An accurate map can be made with the help of overlapping multisite mutants (8, 78). In crosses between a given marker and a multisite mutant, failure to obtain an appropriate class of recombinants indicates that the marker is located in the region covered by the multisite mutation. If several multisite mutants are available for tests, markers can readily be classified into groups which belong in different regions of the genetic map. Order within each group can be determined by intercrossing the group members.

The third and most reliable way of mapping a locus is by means of "three-point tests," in which the order of three markers is determined from the relative frequencies of various recombination classes observed in experiments involving all three. This method can be applied most readily when one has a good marker that is closely linked with the locus being studied (37). Chase & Doermann (21) and Edgar (52), employing three- and four-point tests, have confirmed the linear order of some phage markers previously mapped by Benzer by the first two methods described above (7, 8, 9).

An additional method has been developed for the ordering of mutant sites in the pan-2 locus of Neurospora [Case & Giles (18, 19, 65)]. It relies on the general preponderance, among pan-2 recombinants, of classes representing crossing over with outside markers—an effect, however, which is not always observed [e.g., data of Freese (59)]. In ordering mutational sites within the hisD locus of S. typhimurium by means of transduction tests, Hartman et al. (79) determined average frequencies of recombination between a series of D alleles and a number of markers to either side of the D locus.

Limits of a locus.—In Salmonella, and presumably in bacteria generally, there are marked tendencies for genes controlling related reactions to be located next to one another and to be arranged in the same order as the sequence of their biosynthetic blocks. This kind of arrangement makes it possible to determine, with a reasonable degree of certainty, the limits of certain gene loci and to analyze the areas bordering on the boundary line between two adjacent loci.

The best material we have for such analyses is provided by Hartman's studies of the histidine region, where eight loci have been established (76 to 79a). The evidence indicates that locus hisB, which controls imidazole-glycerol phosphate ester dehydrase, and locus hisC, which controls imidazoleacetol phosphate ester transaminase, are adjacent. We know of 34 single-site hisB mutants, representing 31 sites, as well as one multisite hisB mutant; and we have 35 single-site hisC mutants, representing 33 sites. The multisite mutation hisB-22 covers all known sites of the hisB locus. Figure 1, which presents the two loci diagrammatically, shows (because of space limitations) only a few of the sites in each. Findings about the hisB and hisC mutants are as follows: (a) all hisC, but no hisB, mutants are able to carry out reaction B at a significant rate; (b) all hisC markers recombine with and

complement hisB-22 as well as all other hisB markers; (c) none of the hisC markers complements any other; and (d) none of the hisB markers complements hisB-22. Thus, the evidence suggests a sharp dividing line between loci B and C. Mutations at sites on one side of the line affect one enzyme, and can be distinguished by complementation tests from mutations on the other side, which affect another enzyme.

Within the hisB locus four complementation groups have been recognized. It is interesting to find that about 10 per cent of hisB alleles (including hisB-22) only partially complement hisC alleles, although they show normal complementation with mutant genes of the other his loci. (Partial comple-

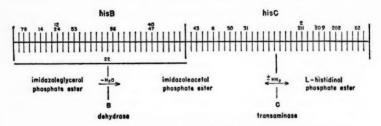


Fig. 1. Diagram of the his B and his C loci of Salmonella typhimurium (according to Hartman).

mentation is indicated by abortive-transduction colonies of especially small size.) The most reasonable interpretation of these and other data Ames [(2); Hartman et al. (78)] seems to be that certain mutations within one locus may affect functioning at an adjacent locus or adjacent loci—a kind of position effect.

Differentiation among alleles.—As soon as multiple allelism had been discovered, geneticists recognized that alleles of the same locus may differ considerably in various ways. Such differences are now being revealed also in microorganisms. One category of differences observed in fungi, bacteria, and bacterial viruses relates to mutation pattern, that is, degree of spontaneous or induced mutability (7, 63, 80). About 60 per cent of auxotrophic and fermentation alleles in S. typhimurium are "mutagen stable": they mutate spontaneously, but their mutation rates are not increased by treatment with any of a large number of mutagens (80, 188).

Allelic mutants may also differ as to nutritional requirements. Hartman (76) observed considerable differences in "leakiness" and response to purines among histidine mutants of S. typhimurium. He found that the histidine requirement of hisA-6 bacteria can be satisfied also by any one of several purines, whereas other allelic mutants can grow only on histidine-supplemented medium. Alleles of a similar nature have been identified for other his loci (78, 79).

In Neurospora, Giles (65) showed that allelic mutants of the ad-4 locus which originate by forward mutations in the wild type may differ in degree of enzyme activity, and that reverse mutants may differ in maximum degree of restoration of enzyme activity [see also (12, 54, 56, 134)]. Some of the recorded differences among alleles of various loci are listed in the seventh column of Table 1.

An interesting pattern of distribution of differentiated sites within a locus was analyzed by Ozeki (40) in one of the adenine-thiamine loci (athA) of Salmonella. Pantothenate satisfies to various degrees the thiamine requirement of the allelic mutants, and they can be classified in three grades according to their growth on adenine-pantothenate medium. Grade I does not grow at all, III grows normally, and II shows heterogeneous growth. Ozeki determined the genetic map order of the nine athA alleles, as follows:

$$8(III)$$
— $7(II)$ — $1, 2, 3(II)$ — $4(I)$ — $18(I)$ — $16(II)$ — $9(III)$.

Apparently the distribution of sites within locus athA is not a random one; sites representing the typical adenine-thiamine phenotype (grade I) are in the central portion of the locus, whereas those representing incomplete biosynthetic blocks are located at the two ends. The findings suggest a polarity in the arrangement of sites, with a gradient from the center toward the ends.

Another example of differentiation among alleles of the same locus is found in Clowes' (23, 24) studies of 13 nonidentical alleles of the cysB locus. The nutritional requirement in all 13 mutants can be satisfied by cysteine. Four of them, however-cysB-18, -24, -25, and -10-are able to utilize thiousulphate instead. Since the cysB locus is closely linked with the try loci, the order of most of the cvsB sites could be determined with a considerable degree of precision by means of three-point tests. The site of cysB-14, which is one of the alleles producing a requirement satisfied only by cysteine, is at the left end of the locus map, and to the right of it, in the following order, are the mutational sites of three cysteine-or-thiosulphate alleles (-18, -24, and -25), seven cysteine-only alleles (-4, -12, -16, -27, -40, -41, -45), one more cysteine-or-thiosulphate allele (-10), and, farthest to the right, another cysteine-only allele (-15). Thus, the sites of these two kinds of alleles are not distributed at random in the locus, nor is each kind segregated by itself, but there is an evident tendency toward grouping, with the two kinds of groups intermixed.

Gots (71) has reported an *ade* locus of *Salmonella* in which mutations in one complementation unit produce strains that are all "leaky," whereas alleles arising from mutations in the second complementation unit have absolute biochemical blocks. Analogous situations may exist in other loci (78, 79).

Differentiation among "identical" alleles.—Growing evidence indicates that mutations at the same site may give rise to several quantitatively different phenotypes. In phage, Benzer (9) has described four phenotypically

distinguishable kinds of alleles (wild type and three mutants) associated with a single genetic site. Streisinger and his co-workers (166) have detected five different alleles at a single site. Work with Salmonella has contributed several instances in which single-site changes have produced three separate phenotypes (76, 79, 100). The existence of a similar multiplicity of single-site mutations in Neurospora was indicated by earlier work of Giles, and his co-workers (44, 64, 66) and has recently been more convincingly illustrated (65).

The number of alternative forms of "identical" alleles should be limited if single sites in reality represent single nucleotide pairs [see (9)]. The template hypothesis of protein synthesis [for recent discussion see Crick (27)] predicts that different changes at a single site will be reflected in specific, restricted alterations in protein structure. Determinations of amino acid sequences in normal and abnormal hemoglobins (84 to 90), although they do not prove the template hypothesis, at least encourage that viewpoint.

The question of variation among "identical" alleles may be partially elucidated by studies of mutagenesis with certain chemicals, such as those carried out by Benzer & Freese (10, 61). Also instructive, in some material, would be back-mutation analysis of a series of independently derived strains mutant at a single site after treatment with certain chemical mutagens having comparatively specific actions. For full interpretation, however, these genetic tests, although valuable, must be supplemented by equally extensive studies of the fine structure of the protein molecules. A large number of experimental systems are being approached in this manner, and the work can be expected to yield interesting results during the next few years.

Differentiation among loci.—We have cited evidence of differences among sites of the same gene locus with regard to various properties or reactions, such as pattern of mutability and phenotypes of the mutants produced. Differences are also found among different loci, or even among larger segments of the chromosome complex. Loci also may differ in their complementation patterns. In some loci, complementation between alleles is observed frequently, whereas in others it has not been detected even though sufficient material has been available for tests. The observed differences are probably real, rather than incidental to factors involving dominance relationships or to the testing technique, for complementation has been detected by the same technique in different alleles of the same loci when tested with either the wild type or nonallelic mutants.

A striking example of differences among regions of a genome is the variation with regard to multisite mutations in *Salmonella*. Such mutations have been found in several regions analyzed by the transduction method. There are eight multisite mutants among 279 histidine-requiring strains (78), six among 38 proline-requiring strains (Miyake, unpublished data), one among seven *cysA* mutants (29), and one among 65 tryptophan-requiring mutants (unpublished data). None has been discovered among about 120

leucine, 37 threonine, 16 isoleucine-valine, 12 isoleucine, and 14 aromatic-compound mutants.

Thus, in general, multisite mutation seems to be fairly infrequent in Salmonella. An exception to this general rule, however, is found in one of the cystine regions, which includes two adjacent loci, cysC and cysD. Of the 28 mutants isolated so far, 12 (or about 43 per cent) are multisite. In most of them the mutation covers all the known sites of cysD and all but one site of cysC (24, 30).

Mutation.—Back-mutation analyses have shown that different alleles of a locus may differ with regard to both spontaneous and induced mutability. Certain mutants, including most multisite and also some presumed singlesite mutants, are completely stable (9, 79). Certain others mutate spontaneously but are mutagen-stable; that is, no mutagen so far tested, including ultraviolet and x-ray radiation, will elicit an increase in their reversion frequencies (28).

Mutagen-labile mutants are affected to different degrees by treatment with mutagens; several are not affected at all by some of them [specific mutagen stability, Glover (70)]. No mutagen-labile mutant is known at present which is (specifically) stable with respect to ultraviolet radiation. Mutational responses to ultraviolet light appear to be of at least three types. (a) In some mutants, amino acids must be present for postirradiation protein synthesis and, possibly, RNA synthesis, if reversion is to be accomplished [Doudney & Haas (46), Witkin (178, 179)]. (b) In others, reversion does not need a supply of exogenous amino acids, but is extremely sensitive to the temperature of incubation at a critical time after irradiation [Kaudewitz (99)]. (c) In a third group, the two above-named special conditions do not seem to be necessary for the expression of ultraviolet-induced reversions. These types of ultraviolet lability have not yet been correlated with specific responses to chemical mutagens. The very existence of various classes of mutagen-labile genes, however, indicates that the genetic alterations responsible for the mutant state are multiple.

Suppressor mutation.—A considerable body of evidence shows that mutation at another, often distantly located or unlinked locus may partially restore the wild type phenotype in the continued presence of a mutant gene.

It has been assumed in some instances that suppressors accomplish their effect by providing an alternative metabolic pathway. Studies with Neurospora by Lein & Lein (107) were initially interpreted as demonstrating that a suppressor mutation, in phenotypically identical yet complementary acetate mutants, opened up a secondary pathway of acetate production. Close biochemical scrutiny, however, revealed that the suppressor action eliminated the accumulation of inhibitory substances prior to the metabolic block (161 to 164). In the event either of the above-mentioned mechanisms was operative, one would expect a suppressor to be effective for several, or all, of the mutant alleles of a specific locus or several loci in a single

metabolic sequence. Although the categories are not exclusive, in one case the genetic expectation would be for a dominant or semi-dominant suppressor mutation; in the other, a partially or completely recessive suppressor. Suppressors affecting some or all of the alleles of a single locus have been described by Howarth (83) and by Forbes [cited in (94)], but have not yet been analyzed at the biochemical level.

An interesting example of nonspecific suppressor mutation was analyzed in *E. coli* by Hashimoto (38). The analysis indicated that changes in one locus or two closely linked loci are responsible for the occurrence of streptomycin-resistant and streptomycin-dependent mutants, and that the effects of such changes can be suppressed by mutations at another closely linked locus. Sixteen alleles of the suppressor locus were identified, any one of which affected the expression of all streptomycin-resistance and -dependence genes tested.

Some suppressors act on only one, or a few, alleles of a given locus. Table I lists loci for which such allele-specific suppressors have been described. In most cases these suppressors have not been tested for suppression of mutant markers at other loci. When such analyses have been made, however, it has sometimes been found that an allele-specific suppressor can partially restore the wild-type phenotype in a number of mutants associated with seemingly unrelated loci [e.g. (120, 123)]. The metabolic pathways controlled by some "unrelated" loci may not be so distinct as might at first be assumed [example in Glass (69)].

Many other theoretical mechanisms have been proposed to explain suppressor action (82, 163, 174), or may be "constructed" from known examples of metabolic interactions and inhibitions (4, 120, 121, 131). It seems likely that suppressor activities are mediated in various cases by several different mechanisms. The effect of an allele-specific suppressor constitutes one criterion, often a sensitive one, for distinguishing between alleles at a single locus. Suppressors afford, in some instances, a unique opportunity to gain insight into mechanisms of gene function and interactions at the metabolic level.

A recent analysis by Suskind and his co-workers has suggested how an allele-specific suppressor may operate. Neurospora mutant td_{24} forms an enzyme protein abnormally sensitive to inhibition by zinc. The results of immunological tests indicate that the enzyme protein is present in the mutant, and active enzyme can be recovered once purified from the crude extract (167 to 171), yet the organism is unable to synthesize adequate amounts of tryptophan. As Suskind suggests, the function of a suppressor gene "specific" for the td_{24} allele might be to relieve the zinc inhibition by preventing zinc from penetrating into or uniting with the sensitive enzyme. Yanofsky (185) has studied a suppressor in E. coli which restores tryptophan synthetase activity and the protein antigen, both absent in the unsuppressed mutant. Whether this suppression is an example of extreme sensitivity to some agent on the part of an altered enzyme, leading to its denaturation and the

relief of this inhibition, or the result of mechanisms completely different from the one postulated above, remains to be determined.

Modifying genes (modifiers) frequently act in the opposite direction from suppressors, that is, by enhancing the mutant phenotype. For example, gene i in Neurospora, described by Fincham & Pateman (56, 57), is loosely linked to the glutamic dehydrogenase locus and acts, when present, to make the mutant phenotype more extreme and decrease the recovery of glutamic dehydrogenase in crude extracts (54). If Neurospora glutamic dehydrogenase is a zinc-containing enzyme, as the comparable mammalian enzyme is believed to be (62), zinc accessibility could alter the functional enzyme activity in either direction: reducing it below a critical level, as may possibly happen in the case of Fincham & Pateman's leaky am mutant ("modifier" action), or operating to restore it ("suppressor" action). Further, it is clear that altered conditions of culture, or changes in internal environment elicited by an altered genome, may lead not only to apparent variation in enzyme levels [see Wagner & Mitchell (174)] but also to the recovery of entire classes of mutants which would otherwise be lethal or go completely undetected.

Complementation.—Two recessive mutant genes are said to complement each other if the trans heterozygote involving both has a wild-type or near wild-type phenotype, and are said to be noncomplementary if the trans heterozygote has a mutant phenotype. This trans genetic configuration may also be obtained in heterocaryons in fungi, during transient or more stable diploidy or abortive transduction in bacteria, or during mixed infection or infection by a heterozygous particle in phage. Some phage geneticists have used the term "cooperation" rather than "complementation" in reference to this phenomenon. It is assumed that complementary mutants carry mutations which affect different functional units, and that each of them also carries the wild-type unit which will "make up for" the functional deficiency in the other. This is not true of a pair of noncomplementary mutants, where, it is assumed, the mutation in each affects the same functional unit. It has been generally observed that crosses between noncomplementary mutants produce either no recombinants or so few that an especially sensitive technique is required for their detection. Noncomplementary genes behave like alleles of a single gene locus, if a locus is defined as a complex genetic unit governing a specific biological function (such as the production of a particular protein).

But evidence suggested by older studies with higher organisms and now rapidly accumulating in work with microorganisms points to a situation considerably more complex than is implied in the concept of a gene locus as a single unit controlling the production of one enzyme and consisting of many noncomplementary mutational sites. Several analyses have demonstrated complementation between sites which control the same enzyme. We shall mention only three examples that are extensively documented; Table I records numerous other instances of complementation within what appear

to be single loci.

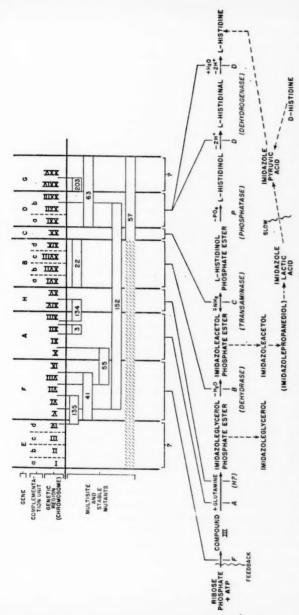
In studies of 123 Neurospora ad-4 mutants (180), 51 complemented at least one other mutant allele. The patterns of complementation indicated the existence of seven distinguishable functional regions. Alleles mutant within any one region did not complement one another. Noncomplementation was restricted to one region in some cases, and extended over two or more regions in other cases. Sixteen different complementation types in all were recognized. On the assumption that complementation is ineffective over a continuous region, their relationships permitted the construction of a complementation map, with the seven units arranged in linear order.

The remaining 72 mutants, that is, more than 50 per cent, failed to complement any other ad-4 mutants. Because of technical difficulties, the locus has not been mapped genetically, and so we do not know whether the linear order of the mutational sites inferred from the complementation map paral-

lels that of a genetic map.

A second well-analyzed case of intralocus complementation concerns 35 alleles of the *Neurospora* locus *pan-2*, which controls the conversion of ketovaline to ketopantoate [Case & Giles (18, 19)]. With regard to complementation, these alleles fall into three groups, six in group 1, three in group 2, and twenty-six in group 3. Those of group 1 complement those of group 2, but not one another, and the group-3 mutants are not complementary to any *pan-2* mutants. According to recombination studies, the sites of alleles of the two principal complementation groups are close together in two relatively distinct regions of the genetic map. This analysis has been extended further by Giles & Case (65).

The third example concerns the hisB locus in Salmonella typhimurium (78, 79). This locus controls the activity of a dehydrase which converts imidazoleglycerol phosphate ester to imidazoleacetol phosphate ester (Fig. 2). Of its 34 known alleles, 18 can be divided into four basic complementation groups: two in group 1, two in group 2, four in group 3, and ten in group 4. The members of each group are not complementary to each other but do complement all members of the other groups. Another 10 alleles complement the members of one or two of the groups but do not complement or are only partially complementary to members of other groups; and 6 alleles do not complement any hisB markers. Recombination data locate the sites of the four basic complementation groups in distinct regions of the genetic map of the locus. Furthermore, the noncomplementary types are mainly the result of single-site mutations scattered throughout the gene locus. The existence of single-site markers which are noncomplementary to different alleles mutant in two or more regions contrasts with the situation in the rII "locus" of phage, described by Benzer (8, 9), and is not in accord with his definition of the complementation regions as "cistrons." We therefore use the term "complementation unit" to designate the basic functional unit. It is possible that some mutations occur at sites especially critical for maintenance of the integrity of a complex gene product, or that mutational sites in one unit exert a "position effect" on adjacent units, interfering with their function or expression.



of some stable mutants, most of them multisite mutants. The bars in each case indicate the extent of the genetic region involved in the Fig. 2. Representation of the histidine region of the Salmonella typhimurium chromosome. The region contains eight gene loci (A-H) Complementation units within loci are designated by lower case letters (a-d). Arabic numerals enclosed by bars are the isolation numbers mutational change. Most of the mutated region associated with mutant his-57 appears to be an inversion (shaded bars). For mapping purposes, the histidine region is divided into a number of subregions (I-XXV) on the basis of tests involving complete transduction (recombination) and abortive transduction (complementation). Each of these comprises several or many individual mutational sites (not shown in the diagram). The map illustrates qualitative features of the histidine region but is not drawn to quantitative scale. For further details for which mutants have been obtained. The biosynthetic reactions with which the genes are directly concerned re plotted beneath the map. There is indirect evidence that the locus of a ninth gene (P), controlling L-histidinol phosphate ester phosphatase, lies between C and D. refer to the text and to Hartman et al. (78, 79).

The demonstration at the genetic level of functional subunits within a gene locus (complementation units) is paralleled (a) by the discovery of structural subunits (probably two dissimilar pairs of units) in the presumed protein-synthesizing particles, ribosomes (58, 173); and (b) by the observation that functional protein molecules are composed of polypeptide or protein subunits, which may be structurally dissimilar (26), similar (62), or a composite of both (91, 92, 93). At present, however, interrelations between the genetical and biochemical findings are not clear. Speculations about various probabilities can be found elsewhere (13, 20, 65, 180). The presence of functional units within single gene loci obviates the necessity for hypothesizing sequential reactions in obscure biosynthetic pathways. Dissimilarities in quantitative phenotype between intragenic complementation units have been noted by Gots (71); and such variations may apply to the C cistrons in phages (95, 112), the three cistrons possibly cooperating in the ultimate formation of a single critical product.

ARRANGEMENT OF LOCI

Early workers with *Drosophila* noticed that "gene loci" are not distributed randomly on chromosomes: that the frequency of close linkage among loci controlling related phenotypic effects is higher than expectancy. In these early cases, the loci were defined by functional tests in heterozygotes. Numerous examples of nonrandom distribution of "loci" in higher organisms have been cited elsewhere (16a, 75, 101, 142). The existence of intragenic complementation has already been discussed; its bearing on the question is obvious. To this problem bacterial research has contributed two further important findings: (a) that functionally related loci are often located close together in bacteria (30, 32, 105, 106); and (b) that the arrangement of such groups of loci on a genetic map, whenever it has been analyzed, coincides with the sequence of the biochemical reactions controlled by the loci.

LOCI CONTROLLING SEQUENTIAL STEPS

Tryptophan series.—In Salmonella, 65 tryptophan-requiring mutants (all of those available in our collection at the time of writing) can be divided into four groups according to their growth requirements and the compounds which they accumulate. The nutritional requirement in 10 group-A mutants can be satisfied by either anthranilic acid, indole, or tryptophan. In 32 group-B and 11 group-C mutants the requirement is satisfied by either indole or tryptophan; the B mutants accumulate anthranilic acid whereas the C do not. The requirement in 12 group-D mutants can be satisfied only by tryptophan. Thus, in the sequence of biochemical steps in tryptophan synthesis, the positions of the blocks associated with the different groups are as follows:

$$\begin{array}{c} - \\ - \\ A \end{array} \rightarrow \text{anthranilic acid} \begin{array}{c} - \\ - \\ B \end{array} \rightarrow \text{indole glycerol phosphate} \begin{array}{c} - \\ - \\ C \end{array} \rightarrow \text{tryptophan}$$

Transduction tests have shown that the loci of the four tryptophan groups are very closely linked, and that their order on a linkage map is tryA-tryB-tryC-tryD. Thus, the arrangement of gene loci corresponds with the sequence of biosynthetic blocks (35, 37). The order of loci was determined by means of three-point tests, each involving two try loci and the cystine locus cysB, which is also linked to the try series.

Experiments with four tryptophan loci in *E. coli* have indicated that they too are arranged in sequence (157, 158). The data of Yanofsky and his colleagues [Crawford & Yanofsky (26)] indicate that the last two steps in tryptophan biosynthesis are carried out on a multifunctional enzyme surface and that the enzyme is composed of two dissociable proteins (steps C and D

above).

Histidine series.—Studies made by Hartman with more than 200 histidine auxotrophs of Salmonelia showed that the eight histidine loci so far identified are all linked together and are arranged in the same sequence as the biochemical reactions they control in the primary pathway of histidine synthesis (76 to 79a). Enzymes controlled by three of the eight loci have been identified. Two other genes have been associated, respectively, with two additional reactions in cell-free systems and a third is implicated in another reaction. The gene order was investigated by means of three methods which produced consistent results. By the first method, the occurrence or nonoccurrence of recombination between the histidine mutants and a series of multisite mutants was observed, the second compared numbers of wild type recombinants resulting from two-point tests, and the third measured average frequency of individual incorporation of one marker in combinations involving a series of other, closely linked markers. Figure 2 shows a genetic map of the eight histidine loci, and the positions of the biosynthetic blocks in their mutants. Although no strains have been found defective solely in L-histidinol phosphatase, a gene for this enzyme is inferred to be present between loci C and D, on the basis of genetic and biochemical tests of a series of his mutants including multisite mutants [Ames (2); Hartman et al. (79)]. It is interesting to note that mutants of the hisE and hisG loci, which are at opposite ends of the histidine linkage group, present novel phenotypes which do not associate themselves readily with any particular single enzyme deficiency. Hartman has suggested that loci his E and his G may represent elements which regulate formation of the enzymes comprising the entire biosynthetic pathway [see also Bonner (13)].

Serine, threonine, isoleucine, and isoleucine-valine series.—In experiments with Salmonella carried out at the Cold Spring Harbor Laboratory, Glanville identified five threonine loci (thrA, B, C, D, E), one isoleucine locus (ile), and four isoleucine-valine loci (ilvaA, B, C, D) [Demerce et al. (38)]. Two serine loci have been identified (serA, B), and one leucine locus (38). Transduction tests have established two linkage groups, one consisting of serB and four of the threonine loci (A, B, C, D) and the other including ileA and the four ilva loci. The available evidence indicates that the four thr loci of

the first linkage group, and all the loci of the second linkage group, are arranged in the same sequence as the biochemical reactions which they control. The reaction controlled by the thrE locus connects the reaction sequences of the first and second groups, but it has not been possible by means of transduction tests to detect linkage between thrE and any of the markers of either group.

Proline series .- Miyake, working with about 200 mutants, was able to identify four proline loci, proA, B, C, and D (38). In the sequence of biochemical reactions involved here (glutamic acid-yglutamic-γ-semialdehyde-) proline), proA and B mutants are blocked between glutamic acid and glutamic-y-semialdehyde, and proC mutants between glutamic-y-semialdehyde and proline. The position of the proD block has not yet been determined. Mivake showed through transduction tests that proA. B. and C are closely linked. Frequencies of recombinants obtained in experiments with markers representing these three loci indicated that proA and proB are adjacent to each other, with proC a little distance to the right of proB. Transduction tests have revealed no linkage between proE and proA, B, or C; but, according to conjugation tests between Salmonella and E. coli, proE is closely linked with proB (Miyake, unpublished data). This finding suggests that in Salmonella the sequential arrangement of loci may be more extensive than can be detected by transduction with P22 phage, which is able to carry only small chromosomal fragments, standard in their sizes and compositions.

DISCUSSION

A large volume of evidence gathered in work with bacterial viruses, bacteria, and fungi favors the hypothesis that a gene locus comprises a segment of genetic material which controls a particular biochemical reaction. Presumably the gene contains the information necessary to determine the structure, and consequently a large measure of the character and specificity, of an enzyme protein (apoenzyme). Within a locus, smaller units can be resolved by recombination, and these "sites" represent the smallest portions of genetic material which can never be split in recombination tests. A change (mutation) occurring within any one of many sites affects the expression of the whole locus. Theoretically, changes at many other sites may not alter the phenotype enough for it to be discerned as "mutant" (isoalleles).

Mutants resulting from changes at different sites of one locus may differ from one another in several respects (stability, reaction to mutagens, temperature sensitivity, nutritional requirements, complementation). As a rule, such mutants have one feature in common: they are changed with regard to one specific function. It also appears that changes occurring at a single site may produce several types of mutants; in one case five types were detected (166). Thus, although a site is the smallest unit of recombination, several different mutational changes may occur within it.

When suitable genetic markers are available it is possible to construct a linear map showing the order of sites within a gene locus, and thus to in-

vestigate the relation between the position of a site and the mutant properties arising from changes within it. Information about such relationships should contribute a great deal toward our understanding of genic structure and function, and one hopes that it will be available soon. That there are functional divisions within a locus, each consisting of a number of adjacent sites, is indicated by the results of complementation studies. The data are still limited and inadequate for definitive interpretation, but the problem is amenable to resolution in the near future.

The findings about structure of loci are in accordance with the view that DNA is the carrier of genetic information in phages and bacteria, and with the model of DNA structure proposed by Watson & Crick (175). The observations about the nature of sites can be accounted for by assuming that a site is represented in the DNA molecule by either one or a few nucleotide pairs. As to the significance of these recent discoveries for our concept and definition of the gene itself, some recent reflections by Beadle (5) may appropriately be quoted:

"In some respects the term <code>gene</code> in the . . . sense [of] a localized unit of genetic material with a specific function . . . is analogous to the term <code>enzyme</code> in chemistry. Biochemists have for many years found it useful to work with enzymes as physically discrete units with specific functions. This was so before their chemical nature was known and before any one of them was isolated in pure form. It remained so after it was discovered that some of them are made up of dissociable subunits. It has continued to be so in spite of evidence that some of the so-called insoluble enzymes are functional only when associated in a particular way with other enzymes. In much the same way the concept of function is useful in defining the gene."

And also:

"That these units can be resolved into subunits capable of separate mutation and of reassociation by some mechanism of intragenic recombination seems an inadequate reason to discontinue calling them genes."

One very striking development of the Salmonella studies is the finding that loci controlling related reactions may be placed close together in the genome, arranged in the same order as steps in the pathway of biochemical reactions they control. Transduction tests have established the existence of this kind of sequential arrangement among the four tryptophan loci, eight histidine loci, five isoleucine and isoleucine-valine loci, and four of five threonine loci. Three of the four known proline loci are closely linked and probably arranged in sequence. Except in a tryptophan series in E. coli, the question of sequential arrangement of loci in other bacteria has not been investigated. The discovery of linkage of genes controlling enzymatic steps in capsule biosynthesis in pneumococci may be indicative of a similar situation.

Thus, in Salmonella, and presumably in other bacteria as well, there is evidence of the control of certain biological functions by blocks of gene loci, and of an intimate relationship among adjacent loci of a genome. The mere

existence of such arrangements shows that they must be beneficial, conferring an evolutionary advantage on individuals and populations which exhibit them. Whether or not adjacent loci had a common origin is irrelevant to the question of their ultimate position, as long as there is a mechanism capable of separating them. With such a mechanism in operation, only selective advantage—during a long series of evolutionary readjustments—could preserve a specific gene arrangement if the genes involved had a common origin, or bring about such an arrangement if they originated in different parts of the genome. It also seems likely that some such force acts to keep the sites of individual loci together, for otherwise it is difficult to see how a locus could include upward of a hundred sites. Probably, in fact, forces which keep the sites of a locus together are considerably stronger than those which maintain specific gene arrangements, because the functioning of a locus as a unit would presumably be disorganized by any appreciable change in either the number or the order of its sites.

Neurospora workers have analyzed four tryptophan and three histidine loci which appear to be analogous to the Salmonella loci as far as their control of biosynthetic functions is concerned. Their locations, however, are in different linkage groups, probably in different chromosomes (3). Therefore it appears that selective advantage as related to gene arrangement is different in different organisms.

It has been suggested [Demerec & Demerec (35)] that one way of explaining the observed difference between bacteria and other groups of organisms is on the assumption that in bacteria many metabolic reactions are carried out by the genes themselves, whereas in higher organisms these reactions are performed by some organelles in the cytosome. Other possibilities have been pointed out elsewhere (76, 79).

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BIOCHEMICAL CYTOLOGY OF MICROORGANISMS1,2

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Biochemical cytologists must consider three fundamental questions which stem from the conception of living organisms promoted by the beautiful work of Jacques Loeb [see Loeb (1)]: (a) What is the anatomy of the cell at the molecular level of dimensions; i.e., what is the spatial distribution of molecules in the cell at any given moment? (b) What movements are made by the cell constituents during metabolic activity and growth; i.e., what paths are traversed in space by the molecular constituents of the cell over a period of time? And linking these questions: (c) What are the causes of the spatial organisation of the cell; i.e., what intra- and intermolecular forces are responsible for the location of the chemical constituents of the protoplasm within the cell and for the transport of these constituents or their precursors or products between the cell and its environment? These questions represent the anatomical, the physiological, and the physico-chemical aspects of biochemical cytology, respectively.

Fortunately for the microbiologist, although most bacteria are relatively small they are not built of correspondingly small molecules (2). Since bacteria of normal size are linearly only about a hundred times larger (ca. 1 µ) than the polymers (proteins, nucleic acids, polysaccharides, lipides) of which they are made (ca. 10 m μ), it would be as impossible for them to contain scale model subcellular organelles (such as endoplasmic reticulum and mitochondria), corresponding closely to those of animals and plants (3), as it would be for the rooms of a doll's house to be built of full-sized bricks and boards. The restriction of complexity in small cells imposed by this effect of scale greatly simplifies the experimental approach to the fundamental aspects of biochemical cytology; and we shall take advantage of this simplification by focussing attention upon bacteria of normal size, only considering observations on larger cells such as those of yeasts or of polycellular organisms inasmuch as they can shed light upon bacterial cytology. As we must follow a linear course of argument it will be expedient to consider the three aspects of our subject in the order most characteristic of development in natural science: configuration (anatomy); process (physiology); and connection between configuration and process (physics and chemistry). It is hoped that those readers who recognize weaknesses in the selection and treatment of the research discussed in the following pages will be sympathetic towards the difficulties confronting the reviewer of this vast new subject.

¹ The main survey of the literature cited in this review was concluded in December, 1958. A few more recent papers have also been cited.

² The following abbreviations will be used: ATP (adenosine triphosphate); DNA (deoxyribonucleic acid); DPN, DPNH (diphosphopyridine nucleotide and reduced form); ETP (electron transfer particle); RNA (ribonucleic acid).

ANATOMY

The range of dimensions given by Palade (4) for mitochondria is 0.2 to $0.5 \,\mu \times 0.3$ to $5 \,\mu$. This is to be compared with the range 0.2 to $2 \,\mu \times 0.3$ to $5 \,\mu$ embracing the cells of most bacteria (5). It follows that the division of bacteria into subcellular units is analogous, from the point of view of molecular size, to the analysis of mitochondria into submitochondrial membranes, particles, and molecules (2).

MAIN SUBCELLULAR ORGANELLES: DEFINITION AND NOMENCLATURE

Protoplasmic organelles.—The deoxyribonucleic acid (DNA) of bacteria, identified by comparing electron micrographs with corresponding stained preparations, appears to be capable of a wide range of distribution within the cell dependent upon the physiological state, the method of fixation, and subsequent treatment [for excellent reviews see Robinow (6) and Maaløe & Birch-Andersen (7)]. Phase contrast or anoptral contrast microscopy in media of which the refractive index is raised by the addition of concentrated gelatin or serum albumin shows the DNA of living Escherichia coli to exist in a region of lower refractive index (dry weight concentration) than the bulk of the protoplasm [Mason & Powelson (8); Mitchell (9)]. Caro, van Tubergen & Forro (10) have shown by radioautography that the distribution of radioactivity among cross sections of cells of a thymineless mutant of E. coli grown in tritium-labelled thymidine corresponds in position to the "nuclear region" seen in electron micrographs of similarly treated cells, thus confirming the conclusion that the DNA is localised in the nuclear region. Although the margins of the nuclear region appear to be quite sharply defined in electron micrographs, Birch-Andersen, Maaløe & Sjöstrand (11) pointed out that no well-defined membrane is seen separating the nuclear region from the cytoplasm. This conclusion is confirmed by the fact that no reflection of light is visible from the margin of the nuclear region in anoptral contrast microscopy of living E. coli (9). Although most of the information concerning the nuclear region comes from studies of E. coli, the observations upon other organisms [reviewed by Robinow (6); Maaløe & Birch-Andersen (7); and Bradfield (12)] indicate that bacteria do not possess nuclear membranes like those of plant and animal cells (13), and there is at present no evidence to suggest that the DNA is surrounded by an osmotically functional membrane such as that which is assumed to be present in Staphylococcus aureus by Webb & Clark (14). The cause of the separation of the nuclear region from the rest of the protoplasm is not known. It may be attributable to cohesion between the components of the cytoplasm or to cohesion between components of the nuclear material, or to both. The isolation of nuclear bodies from naked protoplasts of Bacillus megaterium by Spiegelman, Aronson & Fitz-James (15) shows that the nuclear components cohere under certain conditions, but it is not certain whether the conditions under which the nuclear bodies are isolated correspond at all closely to those in the intact cell. The size of the nuclear region in electron micrographs of thin sections corresponds to a sphere 0.2 to 0.3 μ in diameter in staphyloccoci (12, 16) and to a sphere ca. 0.4 μ in E. coli (17), and would thus represent only about 5 per cent of the volume of the cell (see p. 412). The overall length of the DNA chain is of the order of 1 mm. per cell (7) and it must therefore be tightly coiled so as to be accommodated in a space that is only a fraction of 1 μ across. The effective dry weight density of the DNA in the nuclear region is of the order of 20 per cent, assuming a DNA content corresponding to 1 per cent of the cell dry weight.

The electron micrographs of ultrathin sections of bacteria of normal size do not show any structures resembling the mitochondria of other cells [for a review see Bradfield (12)]. No serious claims have been made for the occurrence of an endoplasmic reticulum or cytoplasmic membranes in bacteria, and none of the electron micrographs of bacteria are believed to show

vacuoles analogous to those of plant cells or yeasts.

The organisation of bacterial cells is comparatively simple inasmuch as they contain few organelles of dimensions above $20 \text{ m}\mu$. After the nuclear region, volutin granules, lipide droplets, and the basal granules of flagella, the most conspicuous differentiated regions of the cytoplasm shown in electron micrographs [Maaløe & Birch-Andersen (7); Bradfield (12); Chapman & Hillier (18); Birch-Andersen & Maaløe (19)] are the probably ringshaped "peripheral bodies" which occur just inside the edge of the equatorial ring or ingrowing transverse septum. In electron micrographs of thin sections the peripheral bodies appear to be electron-transparent roughly circular spaces containing small dense granules. Judging from the size of the peripheral bodies in electron micrographs of thin sections they would not constitute more than 2 per cent of the volume of the cell. The rest of the cell appears to consist mainly of a matrix of components which are not resolvable, in which are imbedded small particles 10 to 20 m μ in diameter.

Plasma membrane, cell wall, slime layer, and capsule.—The definitions of the three main envelopes of bacteria, which are often attributed to Knavsi (20) or Henrici (21), were originally stated very clearly at the beginning of this century by the plant physiologist Fischer (22, 23). He showed that although the protoplasm of living bacteria appeared to behave as a liquid, it would retract from an external cell wall (Zellhaut) in media of which the osmotic pressure had been raised by salt or sucrose but not by glycerol, urea, chloral hydrate, or other lipide-soluble solutes. The protoplasm thus behaved as if it were covered by an invisible, flexible, semipermeable envelope, the plasma membrane (Plasmaschlauch) having the permeability properties of the lipoid membrane proposed at that time by Overton (24). The cell wall retained its original shape during the retraction of the protoplast and, although extremely thin (estimated to be 20 mu thick in Bacillus anthracis). evidently more porous to small molecular weight solutes than the plasma membrane, and without visible fine structure it was considered by Fischer to be rigid enough to be responsible for the shape of the protoplast that it contained under a hydrostatic pressure of several atmospheres, Although Fischer was unable to observe plasmolysis in most Gram-positive bacteria, and wrongly attributed this to the high permeability of their membranes, he was correct in inferring from other considerations that a cell wall and plasma membrane were present in most bacteria whether Gramnegative or Gram-positive. Outside the cell wall, he described a slime layer (Gallerthulle) or capsule (Kapsel) which might be formed under certain conditions. He considered that bacteria did not possess nuclear membranes. The criteria by which Fischer defined the main cell envelopes of bacteria were functional more than directly anatomical and are the same as those which are mainly used to define these subcellular units today [see (25, 26)]. His observations were confirmed by a number of studies of the plasmolysis of bacteria [Ellis (27); Swellengrebel (28, 29); Garbowski (30); Eisenberg (31); Vahle (32); Hölling (33); Raichel (34); Elo (35); James & Gebicki (36)], including certain Bacillus species [Knaysi (37); Robinow & Murray (38); Imsenecki (39); Kuczynski-Halmann & Avi-Dor (40)]; by the microdissection experiments of Wámoscher (41); by the observations of King & Beams (42) on the fluid consistency of the cytoplasm of living Spirillum volutans; and by the independent discovery of Tomcsik & Guex-Holzer (43) and of Weibull (44) that the controlled removal of the cell wall from B. megaterium with lysozyme allows the protoplast to emerge as a sphere. The demonstration of the existence of a membrane of low permeability near the surface of an unplasmolysable Gram-positive bacterium [Mitchell (45)], and Weibull's important discovery that the naked protoplast of B. megaterium could be stabilised by substituting a suitable isotonic medium for the cell wall (44) has led to the definite identification of a plasma membrane, possessing the essential characteristic of semipermeability, at the surface of the protoplast of Gram-positive bacteria [Weibull (46, 47); Mitchell & Moyle (48, 49, 50)]. Weibull (51, 52), Salton (53), Mitchell & Moyle (25), Weidel (54), and Mitchell (2) have reviewed the literature relevant to the definition of cell wall and plasma membrane. Duguid (55), Tomcsik (56), and Wilkinson (57) have reviewed the relevant literature on capsule and slime layer.

In view of the remarkable priority of Fischer's studies of bacterial cytology, his nomenclature has been adopted in this review; and it is hoped that a wider knowledge of Fischer's work may promote the general adoption of his nomenclature. Opinions appear to differ as to the advisability of regarding the plasma membrane as a protoplasmic constituent. In this review, for reasons which will become apparent later, we shall regard the plasma membrane as the outer region of the protoplasm and will refer to the inner region, which it contains, as the "endoplasm."

MOLECULAR ANATOMY

The question with which we are concerned in this section is the pattern of distribution of molecules in the cell, particularly with respect to their relative accessibility from the outer medium. The small size of bacteria greatly favours the accessibility of cytoplasmic components at the surface of the protoplast because the ratio of the area to the volume of a solid figure of given shape is inversely proportional to the linear dimensions. Some geometric data relevant to bacterial structure are given below, using a sphere $1.0~\mu$ in diameter to represent a staphylococcus (S) and a cylinder with hemispherical ends, $1.0~\mu$ in diameter and $3.0~\mu$ in overall length, to represent a coliform organism (C): Volume S $(0.52~\mu^3)$, C $(1.09~\mu^3)$; surface area S $(3.14~\mu^2)$, C $(9.42~\mu^2)$; dry weight, assuming a dry density of $\frac{1}{3}$, S $(17.45 \times 10^{-14}$ gm.), C $(36.46 \times 10^{-14}$ gm.); volume of surface layer $10~\mu\mu$ thick, S $(ca.~0.03~\mu^3)$, C $(ca.~0.09~\mu^3)$; weight of this surface, assuming density of one, S $(ca.~3 \times 10^{-14}$ gm.), C $(ca.~9 \times 10^{-14}$ gm.); number of spheres of diameter $5~\mu\mu$ (mol. wt. ca.~100,000) packing hexagonally in surface, S (145,000), C (435,000); number of spheres of diameter $5~\mu\mu$ in great circle circumference ca.~6.30.

Cell wall.—The work on the molecular structure of bacterial cell walls has been excellently reviewed to 1956 by Cummins & Harris (58); Cummins (59); and Salton (53). Some of the more recent work was described by Park & Strominger (60); Work (61); Salton (62); Weidel (54); Salton & Shafa (63); and McQuillen (64). This work has shown that the cell wall of bacteria such as E. coli and staphylococci represents between 20 and 30 per cent of the dry weight of the cell. In E. coli this would be expected to give a layer about 25 mµ thick and in staphylococci the layer would be rather more than this thickness, assuming the degree of hydration to be the same as the whole cell. On acid hydrolysis the cell walls of Gram-positive bacteria yield up to 3 per cent lipide, invariably glucosamine, the acidic hexosamine muramic acid [Strange (65); Kent (66)] (and variably some other sugars), invariably alanine, glutamic acid, and either lysine or diaminopimelic acid [Work & Dewey (67)] or both (and variably glycine, aspartic acid, or serine). The cell walls of Gram-negative bacteria, on the other hand, contain some 10 to 20 per cent lipide (of which a large proportion can be extracted only after acid hydrolysis), invariably glucosamine, muramic acid, diaminopimelic acid (and variably some other sugars), and a range of amino acids characteristic of normal proteins. Weidel, Koch & Lohss (68) showed that about 80 per cent of the weight of the cell wall of E. coli could be extracted in 90 per cent aqueous phenol. The extracted cell walls were observed by phasecontrast microscopy to be extremely thin but to have retained their normal shape and rigidity. This material was characterised by a high content of sugars, lipides, and a few typical amino acids, and was called the lipopolysaccharide layer (68). Weidel & Primosigh (69) have now shown that the lytic principle of the T phages, either when acting upon whole cell walls or upon the lipopolysaccharide layer, liberates a soluble complex which, on hydrolysis, yields diaminopimelic acid, glutamic acid, alanine, glucosamine, and a substance identified chromatographically as muramic acid. The action

of lysozyme on the cell walls of $E.\ coli,\ Salmonella\ gallinarum$, a psychrophilic strain of Pseudomonas and $Chlorobium\ thiosulphatophilum$ has been shown by Salton (70) to liberate similar soluble complexes. It has therefore been suggested that the cell wall of Gram-negative bacteria consists of a thin rigid network of mucocomplex, similar in composition to the cell wall of Gram-positive organisms, and that this complex is covered on the outside by a plastic film of lipoprotein. Referring to p. 409, the thickness of the mucocomplex layer would have to be ca. 5 m μ and that of the lipoprotein ca. 20 m μ if they were both continuous and contained 70 per cent water. This duplex structure would be partly in accord with the recent electron micrographs of thin sections of $E.\ coli$ in which Kellenberger & Ryter (71) have resolved the cell wall into two lines 2 to 3 m μ thick separated by a space of the same thickness, but one would have expected the outer line to be thicker than the inner one.

As the T phages of E. coli, lysozyme and growth in presence of penicillin all appear to break (or halt the formation of) the bonds which unite the components of the mucocomplex (lipopolysaccharide of Weidel) in the cell walls of both Gram-positive and Gram-negative bacteria [Weidel (54); Salton & Shafa (63); Weidel & Primosigh (69)]; it has been presumed that the presence of the extra lipoprotein component in the cell walls of the latter group constitutes the main difference between the cell walls, and accounts for the differences between the relative sensitivity of Gram-positive and Gramnegative bacteria to penicillin (63, 69). However, the recent excellent work of Baddiley and his collaborators (72, 73) has renewed interest in the polyol phosphocompounds, the presence of which was discovered by Mitchell & Moyle (74). These compounds were shown to be characteristic of penicillinsensitive, Gram-positive bacteria (74, 75), to be located in the cell wall and plasma membrane in the case of S. aureus (74, 76, 77), and to be sensitive to the action of penicillin, the amount rapidly declining from the moment of adding penicillin to a growing culture (78, 79). Further, it was shown that the presence of the polyol phosphocompounds in Gram-positive bacteria was compensated for by a relative deficit of phospholipide as compared with Gram-negative bacteria (75). Baddiley's group have shown that, in addition to the glycerophosphate polymer-which was partially characterised by Mitchell & Moyle (80), and for which they suggested the name glycerol positic acid (77)—there is a ribitol phosphate polymer of similar backbone structure (72). This is linked to glucose and alanine in Lactobacillus arabinosus cell walls (73). Armstrong et al. (73) have proposed that the polymer containing ribitol phosphate which they have found in the cell walls of several Gram-positive bacteria should be called teichoic acid. Since some 30 per cent of the weight of the cell wall of L. arabinosus, Bacillus subtilis, and S. aureus is reported to consist of teichoic acid, whereas none is found in the cell wall of E. coli (73), it is clearly an oversimplification to state that the main difference between the cell walls of many Gram-positive and Gramnegative bacteria is the possession of the lipoprotein layer by the latter. The observation of Piekarski & Giesbrecht (81) that, in electron micrographs of thin sections of B. megaterium the cell wall can be resolved into two components, suggests that in Gram-positive bacteria the cell wall may be duplex.

The duplex (or possibly triplex) structure of the cell wall in a Gramnegative organism was first clearly indicated by the beautiful electron micrographs of the cell wall of Spirillum serpens obtained by Houwink (82). These photographs showed a layer consisting of hexagonally packed spheres about 13 mµ in diameter, closely applied to an apparently homogenous layer. A similar type of structure was observed by Salton & Williams (83) in the wall of Rhodospirillum rubrum, the diameter of the spherical components being about 10 mµ. It is not known whether these two layers may correspond to the supposed mucocomplex and lipoprotein layers of Gram-negative bacteria.

Porosity of the cell wall.—The disruption of the plasma membrane of S. aureus, under conditions which leave the cell wall intact (e.g., using 4 per cent aqueous n-butanol) allows nucleotides and coenzymes, but not proteins, to escape [Mitchell & Moyle (76)]. It was therefore suggested that the effective diameter of the "pores" in the cell wall must be about 1 mu. Other observations support this conclusion. Brenner (84) found that ribonuclease (mol. wt. 12,700; ca. 2.5 mu diameter) would not diffuse through the cell wall plus plasma membrane of intact B. megaterium, but would readily pass through the plasma membrane of naked protoplasts of this organism. Mitchell & Moyle (25) showed that a dextran of mol. wt. 10,000 (ca. 2.5 mu diameter) did not pass into S. aureus after the plasma membrane was broken with aqueous n-butanol. Mitchell (9) also found by studying the degree of contrast obtained in anoptral contrast microscopy that human serum albumin (mol. wt. 68,000; ca. 5 mu diameter) at high external concentration did not fill the space between the cell wall and plasma membrane of living E. coli (strain B) plasmolysed in 0.4 M sodium chloride.

It has been pointed out that a sheet of hexagonally packed spheres 10 $m\mu$ in diameter, like that seen in the electron micrographs of cell walls of spirilla, would have an effective pore diameter of 1.3 $m\mu$ if the spheres were

close packed (25).

There can be little doubt that the cell wall of most bacteria is a fairly porous structure which not only supports the surface of the plasma membrane against a pressure of up to 30 atm. but also acts as a molecular sieve, preventing hydrophilic solutes of mol. wt. 10,000 and above (or diameter above $2.5 \text{ m}\mu$) from leaving the protoplast or reaching its surface from outside the cell wall (25). In spite of the complexity of structure of the cell walls of different bacteria there seems to be one structural element which is common to most if not all of them, namely the so-called mucocomplex polymer containing glucosamine, muramic acid, alanine, glycine, and lysine or diaminopimelic acid.

Plasma membrane.—In the intact cell, the inner margin of the plasma membrane is in direct communication with the complex constitutents of the cytoplasm, representing about a 20 to 30 per cent solution by weight [Barer (85)]. The inner limit of the plasma membrane is therefore difficult to define. In electron micrographs of thin sections of E. coli, Birch-Andersen & Maaløe (19) were unable to distinguish an inner surface of the plasma membrane. Similarly, in Bacillus cereus, Chapman & Hillier (18) concluded that there was no evidence for the existence of a membrane at the surface of the protoplast. One of the main preoccupations of the electron microscopist is, of course, to sharpen the discontinuities of mass density so as to observe details. In the last three years the surface of the protoplast has been described as showing a plasma membrane in S. aureus (12), B. cereus (86), S. serpens (87), in E. coli (71) and in naked protoplasts of B. megaterium (88). It must not be forgotten, however, that these organisms have been fixed with osmic acid or potassium permanganate [which gives particularly good results (89)], and extracted with organic solvents. In view of the observations of Revel, Ito & Fawcett (90) that electron micrographs of thin sections of permanganate-fixed myelin figure membranes (composed only of phospholipide) closely simulate natural membranes, the line referred to as the plasma membrane in electron micrographs of thin sections of bacteria may correspond to a lipide or lipoprotein component of this membrane rather than to the whole membrane. The electron microscope studies which failed to show an abrupt inner margin of the plasma membrane (18, 19) may, perhaps, represent the more natural state of organisation of the protoplasm.

To characterise the material of the plasma membrane it is, of course, necessary not only to isolate it from the other constituents of the cell but also to show that the isolated material has not lost or gained components during the isolation procedures. This has proved to be difficult because the plasma membrane is a very labile structure which, on the one hand, tends to enclose and, on the other hand, tends to give rise to small particles which, after the early studies of Sevag, Smolens & Stern (91) and Schachman, Pardee & Stanier (92), were usually thought to represent miniature counterparts of the mitochondria and microsomes of higher organisms. For example, Alexander & Wilson (93) stated in 1956,

it is the submicroscopic granule of about 20 m μ diameter which corresponds most closely to the mitochondrion of multicellular organisms, by virtue of its possession of enzymes involved in the metabolism of succinate and malate as well as a complete and intact hydrogen transport system.

[See also a review by Alexander (94) on bacterial particulate fractions.] When Weibull (95) discovered that the more stable components of the plasma membrane isolated as protoplast ghosts from B. megaterium were rich in cytochromes but were also fairly heavily contaminated by genuine intra-

cellular particles (which are unfortunately very numerous in this organism), it was not certain whether the cytochromes were indeed part of the membrane or whether they were present in the contaminating particles. Weibull found that brief mechanical disintegration of the protoplast ghosts would readily produce small particles, apparently similar in physical properties to those often isolated from disintegrated whole bacteria. The cytochrome components were still sedimented with these particles on the centrifuge, and Weibull therefore concluded that either the contaminating particles were not easily separable from the membrane material, or the cytochromes were an integral part of the membrane itself [see also Stanier (96) for inspired comments on Weibull's impeccable work]. Electron microscopy of the material of the ghost fraction [Weibull & Thorsson (97)] indicated that the membranes did, in fact, contain additional cytoplasmic material often in the form of granules. The problem of characterising the material of the plasma membrane thus broadened into the comparative study of the membrane and particulate fractions [reviewed briefly by Mitchell (2) and by Weibull (52)]. The suggestion (76) that the small particle fraction of mechanically disintegrated S. aureus represented fragments of plasma membrane was supported by the observation that an acid phosphatase, which was bound to the cell but would hydrolyse phosphate esters in the outer medium, was present almost exclusively in the small particle fraction. The small particles, like Weibull's protoplast ghosts, contained the bulk of the cytochrome system; and, in addition, they exhibited a number of oxidative and hydrolytic enzyme activities [Mitchell (98)]. Clearly, the isolation of the plasma membrane from staphylococci by techniques analogous to those introduced by Weibull (44) would be experimentally advantageous for the differentiation between the natural intracellular particles and membrane constituents inasmuch as staphylococci contain relatively few intracellular particles larger than 20 mu in diameter. Mitchell & Moyle found that the cell wall of S. aureus could be cut into hemispherical parts by a controlled autolytic treatment (49) and that, after exploding the cells osmotically, the more stable components of the plasma membrane could be recovered as punctured spheres practically uncontaminated by particles, provided that manipulation was reduced to a minimum. The weight of the membrane material obtained in this way corresponded closely to the weight of the small particles recovered after mechanical disintegration (49) and their enzymic composition was the same (26, 99, 100). After washing with water or salt solutions, however, the membrane material of S. aureus was found to break up and coalesce into particles which closely resembled the small particle fraction in appearance and in behaviour on subsequent washing or fractionation. Practically the same results were obtained using Micrococcus lysodeikticus, and it was therefore considered to be proved that the small particles represented fragments of the plasma membrane in these organisms [Mitchell (2)].

Newton (101) observed, by the ingenious use of a fluorescent derivative

of polymyxin that shows up in fluorescence microscopy, that polymyxin normally attaches itself specifically to a component of the plasma membrane of B. megaterium. After disintegration of the cells the specific polymyxin-binding component was found almost exclusively in the small particles. Weibull & Bergström (102) have now proved beyond any reasonable doubt that the cytochrome components of the ghost membrane of B. megaterium are present in the membrane itself by showing that "calculated in terms of the dry weight of the ghosts the light absorption is less intense in ghosts containing heavy granules than in normal ghosts." In a paper on "The nature of isolated bacterial fractions as related to preparative technique," Carr (103) observed that

ultrathin sections of Azotobacter vinelandii fail to reveal the presence of large particles, other than occasional metaphosphate granules, or small spherical particles of about 20 m μ diameter noted in this study, by Alexander and Wilson [see (104)], and by Hartman, Brodie and Gray [see (105)]. One must question whether these structures exist in living cells, or whether they result from the cumulative effects of isolation procedures subsequent to cell breakage.

The existence of ribonucleoprotein particles 10 to 20 m μ in diameter in living bacteria can hardly be disputed but, whereas enzyme activities were formerly attributed to such particles isolated from Azotobacter and other species (92, 106, 107, 108), Cota-Robles, Marr & Nilson (109) and Tissières & Watson (110) have now obtained evidence from fractionation studies indicating that the enzyme activities are carried by separate particles which are normally a contaminant of the ribonucleoprotein particles. Electron microscopy of the enzyme-carrying particles shows fragments of the bacterial cell envelopes (103).

In spite of the evidence available, a doubt must remain in the minds of biochemists. This was succinctly expressed by Tissières, Hovenkamp & Slater (111) as follows:

The ghost fraction, after lysis of protoplasts from *Bacillus megaterium*, has been found to contain cytochrome pigments [Weibull (95)]. If the ghosts are exclusively formed of cytoplasmic membranes it follows that the cytochrome system is located in this membrane. This is the conclusion drawn by Mitchell and Moyle (99) from their observation that plasma membranes isolated from *Staphylococcus aureus* by controlled autolysis contained the cytochrome system. However, the possibility that some cytoplasmic constituents adhere to the thin membrane cannot so far be excluded.

If cytoplasmic constituents adhere to the membrane specifically in the living cell and are carried with the membrane during its isolation, one may take the view that they should be regarded as integral constituents of the complex sheet defined as the plasma membrane of the living cell. Indeed, in the opinion of the reviewer it is consistent with present knowledge to regard the plasma membrane as a protoplasmic constituent. Ultimately, of course, the

membrane is composed of particles which are the atoms, molecules, and molecular complexes bonded together, partly by covalencies and partly by ionic and secondary valencies, into a coherent laminar fabric.

The plasma membrane is probably in dynamic equilibrium with components of the endoplasm, in particular with the systems responsible for the synthesis of its precursors (25, 112). It will not always be easy to decide which components of this labile fabric may be lost and which may be added as contaminants during the isolation of the small particles or the ghost of the membrane used for analysis. But, in view of the similarity of composition of the small particle and ghost fractions, it seems likely that the plasma membrane material is sufficiently stable to allow a partial separation from the endoplasmic components, provided that manipulation is reduced to a minimum. The evidence given by the enzyme studies described below indicates that the components of the plasma membrane tend to be lost rather than gained during the isolation procedures. The doubt expressed by Tissières, Hovenkamp & Slater (111) must, however, remain for the time being as the skeleton in the cupboard of those who make inferences about the living bacterium from analyses of the products of its lysis.

Chemical composition of small particles and ghost fractions.—The small particle fraction of S. aureus was found to represent about 10 to 15 per cent of the dry weight of the cell and to contain 22.5 per cent lipide and 41 per cent protein [Mitchell & Moyle (76, 99)]. Gilby, Few & McQuillen (113) found that the ghost fraction of M. lysodeikticus represented about 10 per cent of the dry weight of the cell, and contained 28 per cent lipide (80 per cent of it being polyphosphatidic acid), 50 per cent protein, and 15 to 20 per cent carbohydrate. Weibull (114) observed that the ghosts of B. megaterium (strain M) represented 15 to 20 per cent of the dry weight of the cell and contained 13 to 20 per cent lipide; and Weibull & Bergström (102) found 15 to 20 per cent lipide, 65 per cent protein, and 1 to 10 per cent carbohydrate (probably glycogen) in the ghosts of B. megaterium. Most of the lipide of the ghosts of M. lysodeikticus and B. megaterium appears to be phosphatidic acid. The carbohydrate component of the membrane of M. lysodeikticus was found to yield mainly mannose on hydrolysis. The amino acid analysis of the protein of M. lysodeikticus shows a fairly normal amino acid distribution and suggests that the main protein components are not unusual in composition (113). These observations are consistent with the view that the ghosts and small particles represent a lipoprotein component of the plasma membrane in which the amounts of lipide and protein are approximately enough to allow one monolayer of each to cover the protoplast, but it does not, of course, imply that the lipide and protein need be present in separate layers.

Vennes & Gerhardt (115) found 11 per cent ribonucleic acid by weight in the protoplast ghost of *B. megaterium*, but this was not corroborated by Weibull & Bergström (102). Gilby, Few & McQuillen did not find significant quantities of RNA in the protoplast ghosts of *M. lysodeikticus* (113). Mitchell & Moyle observed that the protoplast ghosts of *M. lysodeikticus*, prepared by an autolytic method similar to that described for *S. aureus* (49), contained about 10 per cent of the acid-soluble nucleotide of the whole cell. This nucleotide was readily dissociated from the ghost fraction by lysozyme or by salt (116).

The composition of the ghost component of the plasma membrane is, as would be expected, very different from that of the cell wall.

Porosity of the plasma membrane: the osmotic barrier and link.—The proposal was made ten years ago that the unit which effects the osmotic separation of the cell interior from the external medium should be called the "osmotic barrier" [Mitchell (117)], and this term has recently come into general use. The properties of the osmotic barrier were originally described as being, on the one hand, the passive property of impeding osmotic equilibration of hydrophilic solutes generally, and, on the other, the active properties responsible for the uptake and accumulation of particular hydrophilic metabolites within the cell (117). It was suggested that the substrate-specific active properties of the barrier might be accounted for by the linkage of metabolic reactions in or on the outer region of the osmotic barrier to a specific permeability property, as exemplified by a hypothetical mechanism for passage of glutamate through the osmotic barrier as glutamine (117). The elegant thermodynamic description by Rosenberg (118) of specifically permeable membranes as connecting links, catalysing the transfer between the phases which they otherwise separate, led Mitchell & Moyle (26) to introduce the complementary term "osmotic link" to describe the components of the osmotic barrier responsible for the substrate-specific communication between the cell interior and the external medium (25, 119). It should be emphasised that the terms osmotic barrier and osmotic link are primarily descriptive of function and do not embody any hypothesis concerning the identity of the components which separate the media on either side with respect to certain substrates, but link them with respect to others (26). The different hypotheses which were later introduced to explain the mechanism of osmotic linkage in bacteria will be considered later.

Simple spatial considerations imply that the components representing the osmotic link and osmotic barrier for a given class of solutes must correspond effectively to different areas of the same continuous cell envelope. The position of this envelope for solutes of small molecular weight has been shown to correspond fairly closely to that of the plasma membrane, both by the observations on plasmolysable bacteria discussed above and by the finding that the volume of intact cells or naked protoplasts impermeable to most small molecular weight solutes carrying more than four water molecules, corresponds approximately to that of the protoplast (2, 25, 45, 46, 47, 50). It is possible that, in view of its comparatively hydrophobic properties, the osmotic barrier corresponds to a monolayer of lipide, as in the classical

lipide membrane (120). If this were so, the components causing osmotic linkage would have to be present in this sheet (at least for part of the time). To confer specificity of permeability (or transport) the components representing the link for one solute must, in general, represent the barrier for others. In case it might be thought that the protein constituents of the plasma membrane would necessarily be less effective than the lipide as barrier components, it was pointed out [Mitchell (2)] that the lipide-free protein membrane of the head of the T-even phages has, under certain conditions of temperature and salinity, a very low permeability to hydrophilic solutes of small molecular weight [Anderson (121)]. It is evident that the envelope defined as the plasma membrane includes a more or less continuous sheet of laterally bonded material through which ions and polyhydric organic substances of small molecular weight cannot pass without being deprived of their shell of water. This sheet probably includes lipide and protein, and it

may also contain nucleic acid and even nucleotides.

Enzymic composition of small particle and ghost fraction.—The protein component of the ghost and small particles appears to include most of the enzymes which were formerly classed as insoluble or particulate. Weibull (95) showed that the cytochromes of B. megaterium sedimented with the ghost fraction during differential centrifugation. Mitchell & Moyle (99) found that the ghosts or small particles of S. aureus contained at least 90 per cent of the cytochrome system of the cells. The percentage distribution of other enzyme activities between the membrane (M) and endoplasm (S) were as follows (90, 100, 122): Succinic dehydrogenase, M (>90), S (<10); lactic dehydrogenase, M (80 to 95), S (5 to 20); malic enzyme, M (>90), S (<10); malic dehydrogenase, M (>90), S (<10); formic dehydrogenase, M (>90), S (<10); α -glycerophosphate dehydrogenase, M (50 to 70), S (30 to 50); glucose-6-phosphate dehydrogenase, M (3), S (97); glucose-6phosphatase, M (10), S (90); acid phosphatase, M (>90), S (<10); succinate activating system, M (50 to 90), S (10 to 50); succinyl-R deacylase, M (3), S (97). The total haem, estimated as pyridine haemochromogen, amounted to some 2000 molecules per plasma membrane (2). Storck & Wachsman (119) observed that the ghosts of B. megaterium contained the systems responsible for oxidation of succinate and DL-lactate, while the soluble fraction contained the systems oxidising glucose, pyruvate, citrate, and cis-aconitate. The oxidation of L-malate was catalysed about equally by each fraction and the oxidation of α -ketoglutarate, although catalysed by the ghosts in presence of an oxido-reduction dye, required a factor present in the soluble fraction for reaction with molecular oxygen. Gale & Folkes (123, 124) observed amino acid incorporation in staphylococci treated with ultrasonics and considered the membranes to play a part in the activity of their preparations. It would be hard to judge, however, from the "poached egg" appearance of these partially degraded organisms, how much of the activity should be attributed to endoplasm and how much to membrane. Butler, Crathorn & Hunter (125), and Brookes, Crathorn & Hunter (126) have demonstrated amino acid activation and incorporation by protoplast ghosts of *B. megaterium*. Ultrasonic radiation disintegrated the membranes and solubilised the amino acid activation enzymes. Haugaard (127) found D- and L-lactic acid oxidases in the small particle fraction of *E. coli*. Cota-Robles, Marr & Nilson (109), working with *A. vinelandii*, found that Purified hulls [cell wall plus plasma membrane] obtained by brief sonic oscillation

Purified hulls [cell wall plus plasma membrane] obtained by brief sonic oscillation contain hydrogenase, cytochrome, phospholipid and the requisite enzymes for oxidative phosphorylation. . . . Hulls free of visible granules reduce neotetrazolium with hydrogen and form granules of formazan which confirms the finding of Weibull [see (128)] that the granules themselves are not the site of reduction.

Connell, Lengyel & Warner (129) found the main activity of an amino acid incorporation system in the cell wall plus plasma membrane fraction of A. vinelandii.

The following papers describe the occurrence of enzyme activities in particulate or soluble fractions without reference to the natural distribution in the cell. Alexander (94) reviewed the literature on the particulate enzyme systems of bacteria in 1956. Alexander & Wilson (93) found that in A. vinelandii the particulate fraction contained most of the following enzyme activities: cytochrome oxidase, diphosphopyridine nucleotide oxidase, succinic dehydrogenase, and malic oxidase; while the activities mainly present in the soluble fraction were as follows: fumarase, aconitase, isocitric dehydrogenase, and α-ketoglutaric oxidase. It was noteworthy that the malic oxidase activity was difficult to sediment while the other four particulate activities all sedimented with approximately equal ease. Bruemmer et al. (130) isolated from A. vinelandii an electron transfer particle very similar to that obtained by Crane, Glenn & Green (131) from ox heart mitochondria. This particle exhibited succinic dehydrogenase and DPNH dehydrogenase activity, contained 28.6 per cent lipide, flavin: haem: nonhaem iron: copper in the ratio 1:5: 16:2, and gave the spectrum of cytochromes-a2, b, and a mixture of c4 and c5. At that time the ETP was "conceived as a chemical unit containing the catalytic components in constant molecular proportions firmly linked to one another" (131). It is now known, however, that the ETP of ox heart mitochondria is a small vesicle which represents a fragment of the mitochondrial membrane [Ziegler, Linnane & Green (132)]. It seems probable that the ETP of A. vinelandii is likewise a plasma membrane fragment, and it may be assumed that the constant ratio of activities is a reflection of the uniformity of distribution of enzymes and carriers in the pieces of membrane which are recovered from the disintegrated cells. The observations of Nisman & Hirsch (133) and of Nisman (134) on amino acid activation by fractions of disrupted (protoplasts) of E. coli have shown that all the amino acid activation enzymes are present in a sedimentable fraction. The amino acid incorporation system is also present in this fraction. They do not consider the possibility that this fraction may be derived from the plasma membrane. King & Cheldelin (135), studying pathways of glucose oxidation in Acetobacter suboxydans have summarised some of their results as follows:

All enzymes for glucose oxidation by the pentose cycle exist in soluble (non-particulate) extracts [Hauge, King & Cheldelin (136)]. On the other hand, the particulate fraction which contains most of the cytochromes in the organism catalyzes a one-step pyridine nucleotide-independent oxidation of glucose with the optimum pH of 5.5 [King & Cheldelin (137); Widmer, King & Cheldelin (138)]. δ-D-Gluconolactone has now been identified as the product of D-glucose oxidation catalyzed by the particulate enzyme. An enzyme responsible for the lactone hydrolysis to gluconic acid also exists in the crude particulate fraction. . . . Another enzyme which catalyzed the oxidation of D-glucose to δ-D-gluconolactone with the optimum pH of 8.6 was found in the soluble (non-particulate) extracts. . . . This enzyme was strictly DPN-specific.

Space will not permit the inclusion of further examples.

The functional significance of the rather sharp localisation of a given enzyme either mainly in the membrane or mainly in the endoplasm will be

considered under Physiology (p. 423).

10

Distribution of total enzyme protein between plasma membrane and endoplasm.—If we accept the conclusion drawn earlier that, generally speaking, the particulate enzyme activities of bacteria are derived from fragments of the plasma membrane, the fact that about an equal number of enzyme activities seem to be exhibited by the soluble and particulate fractions would imply that there cannot be a very unequal distribution of total enzyme protein between the plasma membrane and endoplasm. This conclusion may seem, at first sight, surprising or impossible, but consideration shows that it is in accord with what is known about the overall composition of bacteria. The protein of the ghost of the plasma membrane of staphylococci and of B. megaterium represents some 5 per cent or more of the dry weight of the cell (26, 76, 102, 114). In Gram-negative organisms such as E. coli and A. vinelandii it is more difficult to arrive at an accurate estimate of the weight or composition of the plasma membrane because of the presence of the lipoprotein component of the cell wall from which the plasma membrane cannot readily be freed; but it is probably safe to assume that, as in the Gram-positive organisms, the protein of the plasma membrane represents some 50 per cent of the weight of the membrane and some 5 per cent of the dry weight of the cell.

The quantity of RNA in bacteria ranges between about 2 and 50 per cent of the dry weight of the organisms and is dependent on the rate of growth [see e.g. (139)]. In bacteria harvested from normal exponentially growing cultures, the RNA content generally represents about 25 per cent of the cell dry weight although in staphylococci it is lower, being about 10 per cent (139). The excellent work of Wade & Morgan (139) and of Tissières & Watson (110) on the physical state of the RNA of E. coli has established that

when harvested from exponential growth with a mean generation time of ca. 30 min. some 90 per cent of the RNA content of the organism is present in ribonucleoprotein particles. These particles contain 60 to 65 per cent RNA and 40 to 35 per cent protein by weight, and thus constitute some 40 per cent of the dry weight of the organism. The protein of these particles, formerly reported to contain respiratory enzyme activity (92, 106, 107, 108), has been found by Cota-Robles, Marr & Nilson (109) and by Tissières & Watson (110) not to contain enzyme activity after the particles have been purified. Assuming that the composition of the ribonucleoprotein is the same in fast growing staphylococci as in E. coli it would represent about 15 per cent of the cell dry weight. The approximate percentages of the dry weight accounted for by various other components in E. coli and staphylococci, respectively, are as follows: lipide-extracted cell wall, 15 and 20 to 30; lipide plus DNA, 10 and 10; diffusible solutes, 5 and 20. Making the conservative estimate of another 10 per cent to include carbohydrates and other components of the protoplast, this leaves 15 and 15 to 25 per cent of the cell dry weight, respectively, to account for the total protein of the protoplast, of which 5 per cent is in the membrane. We would thus estimate that the ratio of the amount of protein which may possess enzyme activity in the endoplasm to that in the plasma membrane is about two to one in E. coli and between two and four to one in staphylococci. This estimate is, of course, very approximate and should only be taken as a rough guide. As far as the reviewer is aware the data are not available to make a more exact estimate. It is very interesting to note that the enzymic material of mitochondria appears to be approximately equally divided between the soluble and particulate fractions (140, 141, 142). There is no direct evidence to show what proportion of the plasma membrane or of the endoplasm of bacteria may be enzymically active but, at all events, it is evident that the proportion cannot be very dissimilar in the membrane and in the endoplasm.

The weight of a fast growing staphylococcus being at most (see p. 412) 3×10^{-13} gm., the amount of protein would be between 4.5 and 7.5×10^{-14} gm. and, assuming a mean molecular weight of 100,000, this would be equivalent to about 300,000 to 500,000 molecules per cell, of which about 100,000 would be in the plasma membrane. Herbert & Pinsent (143) estimated that a cell of *M. lysodeikticus* (of assumed dry weight 2.5×10^{-13} gm.) contains 10,000 to 20,000 molecules of catalase (mol. wt. 230,000). This cannot, however, be regarded as a guide to the quantities of the other enzymes since the catalase activity of *M. lysodeikticus* is very high. McIlwain (144, 145) has calculated (by comparing the rates of metabolic processes with the probable turnover numbers of the enzymes catalysing them) that the enzyme molecules responsible for each step in the metabolism of the catalytic intermediates, such as occur in coenzymes, may be numbered in units or tens and that the enzyme molecules responsible for each of the successive trans-

formations of energy metabolism must probably be numbered in thousands per cell. When one considers that the number of enzymes present in a bacterium may, as Gale (146) has pointed out, amount to 500 or more, it would seem that many of the 200,000 to 400,000 protein molecules of the endoplasm and many of the 100,000 protein molecules of the plasma membrane of a staphylococcus must be enzymes.

PHYSIOLOGY

The aim of this section of the review is to consider certain aspects of those movements of molecules or chemical components within the organism and between the organism and its environment which constitute the processes of metabolism, of assimilation, and of growth. The main structural element of the cell, relative to which these movements are directed, is the cell wall; for the protoplasm usually possesses little structural rigidity [reviews by Mitchell & Moyle (25); Salton (53); McQuillen (147)]; sufficient, perhaps, to position the nuclear region within the protoplast. The cell wall normally carries out its formative function by containing the plasma membrane, and exerting upon it considerable mechanical forces which determine its shape and counterbalance the internal pressure. The internal pressure may result in part from the equilibrium forces of surface tension which cause the protoplasts of cylindircal bacteria to become spherical when the cell wall is removed (43, 44). It is known, however, that in normal growth media an internal pressure of some 3 to 30 atm, arises from the presence within the protoplast of a high osmotic concentration of certain solutes [Mitchell (45); Mitchell & Moyle (25, 48, 49, 50); Gale (148, 149)]. The internal pressure must be created by the so-called active transport systems, that is to say, by the coupling of certain chemical bond exchanges of metabolism to the uptake, through the osmotic link, of nutrients [e.g., amino acids (40, 48, 149, 150); phosphate (45, 151)] which are accumulated in the protoplast.

TRANSPORT PHENOMENA

Osmotic linkage and cell wall synthesis.—As the cell grows and divides, the cell wall expands and changes shape, controlling the expansion and shape of the protoplast [Graham (152); Puck & Lee (153); McQuillen (154); Fitz-James (155); Lark (156, 157)]. The protoplast, on the other hand, is the seat of the synthetic reactions responsible for cell wall production, for it has been shown that precursors of the cell wall are initially present in the protoplast and are not polymerised direct outside the osmotic barrier [Park & Strominger (60); Armstrong, et al. (73); Thorne (158); Ishimoto, Saito & Ito (159)]. The problem therefore arises as to how the components of the cell wall can be carried through the plasma membrane without making a hole in it, which would let out the internal solutes, while maintaining the tensile strength of the cell wall. Mitchell & Moyle (25) suggested that this might be done by the

presence of molecules of a reversible (lysozymelike) glycosidase, situated in the plasma membrane beneath the growing edge of the equatorial ring (49, 160), which would simultaneously catalyse the penetration of the cell wall precursors through the plasma membrane and polymerise them on the outside. Enzymes catalysing the translocation of chemical groups in this way have been called group-translocases [Mitchell (2, 161); Mitchell & Moyle (162, 163)]. The far-sighted work of Park (164) on the nucleotides which accumulate in S. aureus growing in penicillin-containing media has led to the identification by Park & Strominger (60) of uridine diphosphate acetylmuramic acid peptides corresponding in amino acid composition to the cell wall [see also Ishimoto, Saito & Ito (159)]. They pointed out that these substances are probably the precursors of the mucocomplex, and suggested that they were translocated through the plasma membrane and polymerised on the outside, not by a lysozymelike enzyme as suggested in the work described above, but by a "transglycosylase" (meaning a synthetase). Baddiley and his collaborators (73) have observed the accumulation of cytidine diphosphate ribitol which is presumed to be a precursor of the teichoic acid component of the cell wall of certain Gram-positive bacteria, and a similar mechanism of polymerisation may be involved. It is significant that a chitin synthetase of Neurospora, catalysing the transfer of N-acetylglucosamine from uridine diphospho-N-acetylglucosamine, has been shown by Glaser & Brown (165) to occur in the particulate fraction of disintegrated organisms and to be solubilised by n-butanol. A study by Crathorn & Hunter (166) of the relative rates of uptake of radioactive alanine by the protoplast membrane and cell wall in B. megaterium also appears to imply that the precursors of the cell wall occur first in the plasma membrane.

The work of Fitz-James (155), Murray, Francombe & Mayall (33), and Lark (156, 157) suggests that the peripheral bodies may be involved in cell wall synthesis, but there is no biochemical or cytochemical evidence to confirm this. The observations of Tomcsik and his collaborators (56, 167, 168, 169) imply that the polysaccharide components of the capsule and cell wall of Bacillus spp. are formed in the region of the transverse septa or growing points [see also Wilkinson (57)]. It is therefore possible that precursors of certain of the cell wall components may be produced in the peripheral bodies and others elsewhere in the cell. To account for the fact that cell wall synthesis by the protoplast is deranged or stopped after the cell wall has been damaged or removed (152 to 157), Salton (53) suggested that the synthesis of the cell wall occurs in the peripheral bodies and that these bodies are attached to the cell wall outside the plasma membrane. The electron microscopical evidence reviewed above is not, however, in favour of this explanation. Bearing in mind that, as Robinow (170) has aptly put it, many bacteria consist of "two cells about to become four," the explanation most consistent with the available facts would seem to be that the regions of cell wall extension, which are responsible for the normal morphology of the cell during growth, are determined by the distribution of group-translocases at the interface between the plasma membrane and cell wall in the growing region of the cell near the transverse septa (i.e., close to the peripheral bodies). The translocases, being attached to the inside of their polymerised substrate (cell wall), would be expected to lay it down continuously at the growing point by a process akin to crystal growth, the gradual closure of one septum leading, by a delicate protoplasm/cell wall balance, to the desorption and migration of the translocases to the region of a new equatorial ring or transverse septum and growing point. Removal or disturbance of the cell wall in such a system would be expected to result in removal or disturbance of the translocases attached to it and to the loss or disturbance of cell wall-forming capacity in the protoplast. The isolated cell wall would be expected to carry with it enzymes specific for the cell wall substrate, and this has been found to be the case [Mitchell & Moyle (49)].

Osmotic linkage and nutrient uptake and assimilation.—The studies of Gale and his collaborators on amino acid uptake in streptococci and staphylococci show that the components of the osmotic link, at least for glutamate and glutamine uptake, resemble enzyme systems in catalysing a coupling between substrate transfer and the metabolic systems driven by glucose metabolism, by exhibiting a saturation phenomenon, in their susceptibility to inhibitors, and in other respects [reviews by Gale (148, 149)]. The interpretation which Gale has placed upon the extensive observations of his group on amino acid uptake changed from a largely physical (148) to a largely metabolic one (149, 171), assuming the accumulated "free" amino acids to exist as labile compounds on a polymer or gel structure and not confined by an osmotic barrier for small molecular weight solutes, and back again (172); but an acceptable compromise was not struck. It is noteworthy that although amino acids were found to be accumulated in high concentration in the cells of old declining cultures, relatively little free amino acid 'pool' was found in young rapidly growing organisms (148). Moreover, there appeared to be competition between amino acid uptake and peptide bond formation (173), suggesting that external as well as internal free amino acid might be the direct precursor of amino acyl compounds or peptides (161). Cohen & Rickenberg studying amino acid accumulation in E. coli, adopted a metabolic explanation at first (174, 175), but later invoked membrane transport through a nonmetabolic system (176).

The first metabolite transport system of bacteria to receive detailed study was that responsible for the initial step of phosphate assimilation in S. aureus [Mitchell & Moyle (151); Mitchell (45, 98, 177, 178)]. This system showed all the characteristics of a group-transferring enzyme system: Michaelis-Menten (179) kinetics, pH optimum, very high specificity, catalysis of exchange reaction with analogues (H₂³¹PO₄', H₂³²PO₄', and

H₂AsO₄), inhibition by typical enzyme poisons such as thiol reactors, etc. The simplest interpretation of the permeability and kinetic data was shown to be that the phosphate, being unable to diffuse through the osmotic barrier, enters the osmotic link by a group-transfer reaction catalysed by an enzyme in the membrane, giving rise to the formation of an R-phosphate (more correctly R-phosphoryl) compound at the inner side of the membrane [Mitchell (2, 98, 177, 178)]. The phosphate ion was thus visualised as passing into the osmotic link as phosphoryl and entering the internal medium of the cell, not as phosphate but as a phosphoryl compound, the phosphorylation being coupled to the bond exchanges of glucose metabolism (2, 98). The accumulation of inorganic phosphate in the cell was presumed to arise from the enzymic hydrolysis of the R-phosphoryl compound. This interpretation of the facts was in line with the work of other biochemists concerned with the mechanism of coupling of transport to metabolism, notably the work of Davies & Ogston (180). Danielli had described substrate-specific facilitated diffusion, exemplified by the system catalysing entry of glycerol into red blood cells [see review by Danielli (181)]. Doudoroff (182) has suggested, without giving details as to the possible mechanism, that sucrose phosphorylase might be responsible for allowing sucrose to enter bacteria which appeared, from a comparison of the activities of intact and broken cells, to be permeable to sucrose but not to glucose. Monod (183) described observations which showed that in certain strains of E. coli a system was present which would give rise to the active uptake of the unnatural galactoside analogue thiomethyl- β -D-galactoside, not hydrolysed by β -galactosidase. The activity of the system was shown to be induced by its substrate. A kinetic study revealed that this system-assumed to be the same as that responsible for the entry of normal β-D-galactosides into the cell and thus for controlling their subsequent metabolism-displayed characteristics which were very similar to the phosphoryl transport system of S. aureus. Monod concluded that β -galactosides are carried into E. coli by a substrate-specific system independent of and distinct from β -galactosidase. He suggested that "specific uptake systems exist as a general rule for each type of carbohydrate metabolised by a given organism" (183). Monod did not, however, make any measurements of the permeability of E. coli to sugars or sugar derivatives. Kogut & Podoski (184), and Barrett, Larson & Kallio (185) had previously obtained metabolic evidence for the existence of a system controlling uptake of citrate into the metabolic systems of Pseudomonas, the activity of which was induced by citrate, and similar observations were reported by Davis (186). The direct citrate uptake experiments of MacDonald & Gerhardt (187) have not, however, confirmed the simple citrate transport hypothesis advanced by Davis (186).

It was apparent to those engaged in the study of the bacterial transport systems at the molecular level that, to proceed with the analysis, it would be helpful to adopt the method customary in physics and physical chemistry, namely to formulate hypotheses on the basis of all the available evidence and from that basis to search for the means of experimental corroboration or disproof. In permeability and plasmolysis studies Mitchell & Moyle (26, 48, 49) found that glucose, galactose, and mannose would not diffuse nonspecifically through the osmotic barrier and link of E. coli or staphylococci. As the hexoses could, nevertheless, be rapidly metabolised by the enzyme systems of the protoplast it was deduced that they or their derivatives must pass in through the osmotic link by substrate-specific mechanisms tightly coupled to metabolism (26). It was shown that two different types of coupling were possible. (a) The hexoses might diffuse through the membrane on substrate-specific carriers of the type considered by Rosenberg & Wilbrandt (188). These carriers would be saturated when at the outside of the membrane by the high external concentration of hexose. As long as the concentration of hexose in the cell was low enough for the hexose passengers to leave the carrier, the hexose would continue to flow in, but as the internal concentration approached that which would saturate the carrier the number of passengers carried in each direction would approach equality and the net inward movement would cease. This type of system is thus physically and not chemically coupled to the metabolism of the substrate, and represents a permease-type system as later defined by Monod and his collaborators (189, 190). (b) The hexoses may be carried through the plasma membrane by active transport systems which, as exemplified by the phosphoryl transport system, are assumed to be dependent upon and coupled to the metabolism of the substrate which they transport as a chemical group (26, 98, 178).

The unequivocal demonstration of the presence of the enzymes of terminal respiration and the complete cytochrome system in the plasma membrane of *S. aureus* was taken, in conjunction with the above observations, to imply that the enzymes of classical biochemistry (including the classical catalytic carriers) may be responsible for specific membrane transport, and that metabolism and transport may be integral consequences of one set of catalytic processes. This view was formulated as an explicit hypothesis [Mitchell & Moyle (25)], which, for the sake of identification, we will call the metabolic link hypothesis.

Monod's analysis of the systems of $E.\ coli$ concentrating β -galactosides, under conditions precluding their metabolism (either by the use of unnatural substrate analogues, or of metabolically deficient strains or mutants), was extended very elegantly by Rickenberg, et al. (189). The induction, specificity, and general kinetic and chemical properties of the substrate-specific component of the system catalysing the uptake of β -galactosides were found to be closely similar to the corresponding characteristics of the β -galactosidase, and the two were found to be closely genetically linked. The observation that certain ("cryptic") mutants contain the β -galactosidase

but not the transport system in an active form and that, conversely, certain mutants possess the transport system but not the active β -galactosidase was taken to imply that the β -galactosidase and the substrate-specific component of the transport system are different. The specificity of the transport system and its inducibility, blocked by inhibitors of protein synthesis, were taken to show that it involved a protein for which the name permease was proposed. The permease was defined by two subsidiary postulates: (a) that the transport of the substrate through the permease system requires the transitory formation of a specific complex with the permease; and (b) that the permease is functionally specialised and does not take part in the metabolism of its substrate. The hypothesis was proposed that permeases, as thus defined, were generally involved in the penetration of hydrophilic substrates through the osmotic barrier to the metabolic systems contained in the cell. Cohen & Rickenberg (176) explained their observations on amino acid accumulation in E. coli by invoking amino acid-specific permeases, and accounted for the antagonism between L-valine and L-leucine or L-isoleucine by postulating competition for the amino acid permeases.

The literature relevant to the metabolic link hypothesis was reviewed by Mitchell (2, 161), and it was shown that the function of the metabolically coupled transport of chemical groups, in the cases so far studied, might be adequately accounted for by the activity of normal group-transferring metabolic enzyme systems provided that (a) the active centre of the enzyme system (e.g., hexokinase) was anisotropic so that the group donor (e.g., ATP) must react only from one side while the group acceptor (e.g., glucose) must react from the opposite side; and (b) the enzyme molecules are appropriately orientated in the osmotic barrier, Condition (a) insures that group transfer results in group translocation relative to the enzyme molecule; condition (b) insures that the translocation is directed across the membrane and shows as a macroscopic group transport. This conception of transport by group-translocation was later formulated more explicitly and extended [Mitchell & Moyle (162, 163)]. It was suggested that the problem of specific nutrient uptake is not primarily a problem of substrate permeation as usually understood, for much of the evidence suggests that the substrate which disappears outside appears first as a covalent compound within the cell.

Referring to the consideration of the distribution of enzyme activities in the particulate or plasma membrane and soluble or endoplasm fractions of bacteria and assuming that, in the cases where origin was not considered, the particulate and soluble fractions can be equated with plasma membrane and endoplasm fractions, respectively, the following generalisations appear to be permissible. The cytochrome or electron transport system and the enzymes of terminal oxidation are located in the membrane. Certain enzyme activities occur with fairly equal distribution between the endoplasm and the membrane. For some of these activities both particulate and soluble

enzymes are known, the former being pyridine nucleotide-independent (flavoprotein- or cytochrome-linked) enzymes and the latter being pyridine nucleotide-linked. For example, DPNH oxidase versus certain DPN reductases (or DPN-linked dehydrogenases) as shown by the data of Alexander & Wilson (93); and glucose oxidase versus the DPN-linked δ-Dgluconolactone reductase as shown by the data of King & Cheldelin (135). The distribution of α -glycerophosphate dehydrogenase activity shown by the data of Mitchell & Moyle (p. 420) and of malic dehydrogenase activity shown by the data of Storck & Wachsman (119) may indicate a similar sharply defined membrane/endoplasm distribution of the appropriate oxidase and reductase enzyme pairs. The existence of the appropriate particulate and soluble enzyme pair for α-glycerophosphate/dihydroxyacetone phosphate has been demonstrated in insect flight muscle mitochondria by Estabrook & Sacktor (191), and two malic dehydrogenases, one of them pyridine nucleotide-independent, have been shown by Cohn (192) to be present in M. lysodeikticus. It would seem that these oxido-reduction pairs may operate as hydrogen carriers between the membrane and oxidation reactions within the endoplasm, the DPN-independent component in the membrane being directly accessible to molecular oxygen and possibly catalysing translocation of hydrogen, hydroxyl, and/or electrons. Perhaps the most significant evidence concerns the presence of the activation enzymes catalysing the initial step of succinate and amino acid assimilation or metabolism in the plasma membrane. Mitchell & Moyle (122) have found a succinate activating system producing succinyl coenzyme A in the plasma membrane of M. lysodeikticus which exhibits the same kinetic and inhibitor characteristics as the system causing succinate uptake by the intact cells. The transport of succinate evidently occurs as succinyl and under certain conditions free succinate is released within the cell by the activity of a deacylase found in the endoplasm fraction. The metabolism of succinate does not, however, require the deacylation of the succinyl coenzyme A which passes into the cell, Nisman & Hirsch (133), and Nisman (134) have shown that the amino acid activation enzymes of E. coli occur in a particulate fraction and although as mentioned above, they do not place this interpretation upon their results, it seems likely that these enzymes are present in the plasma membrane and are available to the external medium. This interpretation is strengthened by their observation that there is competition between L-valine and Lleucine or L-isoleucine. Further, Gale's work (171) indicates that in staphylococci external amino acid can be the direct precursor of amino acyl compounds or peptides, and the work of Halvorson & Cohen (193) on the uptake of radioactive amino acids by yeast leads to the same conclusion. The facts available at present imply that succinate and amino acids enter bacteria as acyl compounds by group translocation catalysed by the activation enzymes. As these systems are essentially metabolic, catalysing the first step in the synthesis of protein and other essential compounds, they could not be classed as permeases.

The permease hypothesis was extended in a stimulating but rather exclusive review by Cohen & Monod (190) without any fundamental change of definition. The review hinged upon the specific requirements for the accessibility of nutrients to the metabolic systems of the cell, particular attention being paid to the phenomenon of specific crypticity. Serious consideration was not given to the molecular mechanism of transport, but a general model of the permease was described in the following terms:

In places within the barrier there exist different proteins (the permeases) which are able to form stereospecific, reversible complexes with different hydrophilic compounds. Dissociation and association of the specific complex may occur either on the inside or on the outside of the osmotic barrier. The effect of a permease, therefore, is to activate catalytically the equilibration of the concentrations (activities) of the substrate on either side of the membrane. . . . Many permease systems, but not necessarily all of them, are coupled to an energy donor, the net effect of which is to inhibit the "inside" association reaction. . . .

The difficult problem of the mechanism by which the transport could be driven by "an energy donor" was not approached, and it is evident that the model given by Cohen & Monod corresponds fairly closely to the facilitated diffusion-type system which, it has been pointed out by Widdas (194), can cause coupled transport under certain conditions [see also (2, 195)]. The suggestion (2, 161) that permeases might be more exactly defined as the specific protein components of facilitated diffusion systems is therefore a reasonable one. It has yet to be demonstrated whether any of the systems described in the literature as permeases do, in fact, catalyse facilitated diffusion.

Sistrom (196) has shown by the use of osmotically sensitive forms of a mutant of $E.\ coli$ deficient in β -galactosidase activity that the cells swell when they accumulate lactose, thus demonstrating nicely that the accumulation process is an osmotic one. He observed that there was no swelling in metabolisable sugars, suggesting that there was no surplus accumulation of solutes in the protoplast under normal conditions. Recent papers by Lester & Bonner (197), Avigad (198), Pardee (199), and Rotman (200) shed new light on the system (or systems) causing uptake of galactosides (both α - and β -) and indicate complexities which do not at present seem to be resolvable. A new pathway of lactose utilisation independent of hydrolysis by β -galactosidase discovered by Bernaerts & de Ley (201) in Alcaligenes sp. adds to the new factors to be considered. Informative papers by Ball, Shive and their collaborators (202, 203) on antagonisms between amino acids, keto acids, and peptides during enzyme formation in $L.\ arabinosus$ show that, for example

Each competitive inhibition involving the keto acids, peptides or free amino acids is independent of the other two competitive inhibitions; so the utilisation of keto acid

and peptide does not involve the inhibited site of utilisation of the free amino acid. The formation of an active form of valine for enzyme synthesis by separate pathways from keto and amino acid and peptide affords a plausible explanation for these results.

They conclude that

A common point in the utilisation of amino acid, peptide and keto acid could logically be an activated form or complex of the amino acid, but it does not appear likely that this form of the amino acid is merely free amino acid within the cell.

These observations are in accord with the group-translocation concept of nutrient uptake.

Cohen & Monod (190) drew attention to the considerable repertoire of permease proteins which their hypothesis demands in view of the basic postulate of the separateness of the systems catalysing metabolism from those catalysing transport. But they depended upon the conclusions drawn from the studies of mutants mentioned above, to justify this presumed evolutionary extravagance. It should be noted that the interpetation of the studies of the mutants involves two implicit assumptions which are related to the assumption of the separation of transport from metabolism. First, that specific galactoside transport in different mutants cannot be catalysed by different enzymes [but see Pardee (199)]; and second, that the presence or absence of the transport or galactosidase activity in a given mutant is only an index of the presence or absence of the corresponding protein and is independent of the location of the protein in the cell. It is doubtful whether these assumptions, particularly the latter (see pp. 433-34), are justified.

BIOPHYSICAL CHEMISTRY

The ultimate aim of the biochemical cytologist is to describe the organisation of the cell in terms of the relationships between the movement, the transformation, and the location of the molecular constituents of the protoplasm. It has been pointed out (2) that the growth and maintenance of the organisation of the cell depends upon the entry of the small molecular weight nutrients of the medium into the catalytic, regulatory, and structural polymers of the cell wall and protoplasm. This process is, of course, a spontaneous one as it occurs in a normal living cell. To simplify the analysis of the systems responsible for this spontaneous organising activity, biochemists normally divide them arbitrarily into (i) the so-called energy requiring systems more directly responsible for catalysis of the accumulation of the nutrients of the medium and for the rearrangment of their constituent atoms; and (ii) the so-called energy yielding systems which drive the systems of group (i) by the substrate-specific exchange of covalent bonds between pairs of molecules [Green, Stickland & Tarr (204); Dixon (205)]. The energy requiring systems of the cell, driven by the energy yielding ones, catalyse (by an overall spontaneous process) the bringing together within the cell of groups of atoms and molecules which are generally present in relatively dilute solution in the medium, and it has been customary to divide the work done by these systems into: (a) osmotic work, such as is done by active transport of a solute through a natural membrane against an electrochemical gradient; and (b) chemical work such as is done in linking carbon atoms together covalently. The usual definition of active transport as the movement of a solute (S) up a concentration or activity gradient can be misleading because consideration is given to the process which is driven (which is apparently active) but not to the system driving it. Since no chemical component can pass macroscopically up an activity gradient spontaneously, it follows that in the process of active transport as usually understood the component S appears to move up an activity gradient by undergoing transformation to component Y which, having moved down an activity gradient by diffusion, may (or may not) be reconverted to S; for, at the molecular level a flow can only occur by thermal movement or diffusion. Thus, it is Y that undergoes the transport (by diffusion) which is generally described as the active transport of S. There can, in fact, be no fundamental difference in molecular mechanism [see Glasstone, Laidler & Eyring (206); Guggenheim (207)] between, on the one hand (a) a process which leads to the substrate-specific capture of a chemical component on one side of a membrane (which may lead to its subsequent release at a higher concentration on the other side); and on the other hand (b), a process which leads, for example, to the substrate-specific (enzyme-catalysed) capture of a carbon-containing compound by formation of a C-C bond in exchange for a C-S bond [Davies & Ogston (180) have given an excellent discussion of this type of question]. Considerations such as the above represent the justification in physical terms of the view derived from knowledge of the molecular antaomy and physiological behaviour of bacteria, that the plasma membrane by containing the requisite enzyme and carrier systems anisotropically orientated, may catalyse the spontaneous uptake of nutrient, output of end-products and external deposition of cell wall in an organised manner. This view, however, hinges upon the conception of an anisotropic array of the enzymes and carriers directing the organised movement of their substrates by facilitated diffusion and group-translocation. It has been recognised for some time that the arrangement of the separate enzyme proteins in multi-enzyme systems may profoundly influence the biochemical and physiological activities of these systems [reviews by Schneider & Hogeboom (208); Lehninger (209); Chance (210); and Ernster (211); and see, e.g. (131, 132, 212 to 217)]. The fact that the enzymes and carriers of the particulate or membrane fractions of disintegrated cells can be solubilised, some readily, e.g., cytochrome-c [see Schneider & Hogeboom (208)] and others only after treatment of the particulate material with organic solvents such as acetone [e.g., succinic dehydrogenase (218)] or lecithinase [e.g., choline dehydrogenase (219) and α-glycerophosphate dehydrogenase (220)], implies that the proteins are mainly associated with the lipide components of the membrane and with each other by residual valencies. The beautiful demonstration by Tissières (221) and Keilin & King (222) of the reconstruction of succinic oxidase from soluble succinic dehydrogenase and a particulate cytochrome system preparation shows that the association between the enzyme and the complex in which it usually resides is a reversible phenomenon. Massey (223) has shown that α-ketoglutarate dehydrogenase consists of a reversible association between lipoate dehydrogenase (diaphorase) and a second protein component catalysing the reduction of lipoate by a-ketoglutarate. The two protein components, dissociable by 2.5 M urea, are normally tightly bonded together, the lipoate probably lying between them. A similar situation has been shown to exist in the case of some of the enzymes linked by DPN [reviews by Velick (224); and Mahler (225)]. The anisotropic characteristic of the transfer of hydrogen to or from the pyridine ring catalysed by an enzyme pair indicates their anisotropic situation with respect to each other and to the coenzyme (224, 225).

The above and other considerations outside the scope of this review imply that the enzyme and carrier components of the particulate multienzyme systems, and presumably of the membranes to which they normally
belong, are specifically bonded to each other and to lipide components by
residual valencies in much the same way as antibodies are bonded to antigens or as neighbouring parts of the polypeptide chain of a given globular
protein are bonded internally to each other [Waugh (226)]. Further, the
specificity of the bonding does indeed appear to give rise to an anisotropic
arrangment of the active centres of the enzymes and carriers.

The complex spatial interrelationships of the protein components of the protoplasm (including the plasma membrane) which the anatomical, the physiological, and the physicochemical evidence appears to show, poses a genetic question which is of particular interest in considerations of the possible causes of crypticity. The specific interrelationships of the enzyme proteins must be determined by the properties of their surfaces. The properties and configuration of the surfaces of proteins are determined by the amino acid composition and sequence, as exemplified by the haemoglobins [Waugh (226); Itano (227); Hunt & Ingram (228)]. It has therefore been suggested (229) that there may be two partially independent regions of protein catalysts which determine physiological activity: the part of the amino acid sequence and associated secondary bonding representing the active centre, determining catalytic activity; and the part of the amino acid sequence and associated secondary bonding, determining the site at which the catalyst becomes anchored within the organisation of the cell. The genetic consequence of this would be that mutations affecting the amino acid sequence or composition of these proteins might be divided into two partially independent types: those causing change of catalytic activity by changes in the active centre region of the amino acid sequence; and those causing a change of location of the catalytically active protein in the cell by changes elsewhere in the polypeptide chain. This principle is supported by the observation of Benzer (230) that the mutations which affect the catalytic activity of an enzyme are localised in a certain region of the corresponding polynucleotide chain, while leaving silent areas elsewhere. The application of this principle to the occurrence of crypticity is obvious, since the location and proper orientation of a transport system in the plasma membrane is a prerequisite for its functional activity. We may conceive in similar terms the mechanism whereby bacteria may have evolved not only their chemical repertoire in terms of the individual catalytic activities of their constituent enzymes, but also the complex spatial organisation of their components which is responsible for the directiveness of their assimilatory and synthetic activities, and for the generation of the cell as we see it.

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FERMENTATIVE METABOLISM^{1,2}

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A number of fermentations and the enzymes involved in these processes, which have been described in the period from January 1958 to January 1959, are discussed in this review. Some material on the fermentation of nitrogencontaining compounds which appeared prior to this period has been included, since it provides a number of interesting examples of fermentations in which energy-yielding reactions occur other than those involved in the metabolism of pyruvic acid and supplements the data legitimately included. The topics discussed in this review include the fermentation of pentoses, uronic acids, histidine, glutamic acid, glutamine, glycine, creatinine, purines, and pyridine derivatives. Papers describing the aerobic degradation of some of these substrates have been included for comparative purposes. Descriptions of the aerobic fermentation of a number of other compounds by organisms isolated by enrichment culture techniques have also been included.

CARBOHYDRATES AND CARBOHYDRATE DERIVATIVES

Pentoses.—The enzymes involved in the anaerobic fermentation of the pentose L-arabinose by Aerobacter aerogenes and Lactobacillus plantarum have been purified and described in detail during the past year. The fermentation of arabinose and xylose by lactic acid bacteria was studied by Fred, Peterson & Anderson in 1921 (27). The authors isolated and named the organism responsible for the fermentation, Lactobacillus pentosus. This organism was later identified as a species of L. plantarum. Lactic acid and acetic acid are formed in approximately equal ratios as fermentation products and account for 90 per cent of the pentose fermented (27). Lampen, Gest & Sowden (46) and Rappoport, Barker & Hassid (67) demonstrated that C-1 of D-xylose (46) or L-arabinose (67) is converted to the methyl group of acetic acid by L. pentosus (46) and L. pentoaceticus (67).

A number of enzymic reactions involved in pentose metabolism have been demonstrated by Lampen & Mitsuhashi (47, 53), using extracts of L. plantarum; however, the series of enzymic reactions involved in the conversion of pentoses to acetic acid and lactic acid has only recently been elucidated by Horecker and his co-workers. The individual enzymes have been purified, and it has been possible to define the substrate specificity of the

¹ The survey of the literature pertaining to this review was concluded in January, 1959.

² The following abbreviations will be used: ADP (adenosine diphosphate); ATP (adenosine triphosphate); DPN and DPNH (diphosphopyridine nucleotide and reduced form); ITP (inosine triphosphate); TPN and TPNH (triphosphopyridine nucleotide and reduced form); UDPG (uridine diphosphoglucose).

enzymes, as well as to characterize the reaction products (11, 12, 13, 22, 33, 34).

The fermentation of arabinose by A. aerogenes has been investigated by Simpson, Wolin & Wood (75), who found that the conversion of arabinose to xylulose-5-phosphate occurs by the same enzymic reactions as in L. plantarum. However, the subsequent fate of p-xylulose-5-phosphate differs in the two fermentations.

L-arabinose isomerase, which catalyzes reaction A in Figure 1, has been

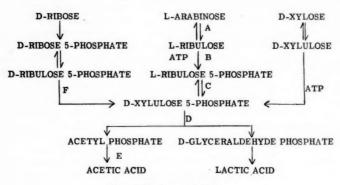


Fig. 1. Pentose metabolism.

- A. L-Arabinose isomerase
- B. Ribulokinase
- C. L-Ribulose-5-phosphate 4-epimerase
- D. Phosphoketolase
- E. Acetokinase
- F. p-Xylulose-5-phosphate 3-epimerase

purified about eightfold from extracts of *L. plantarum* (34). The enzyme is very specific for both reactants. A variety of aldopentoses and ketopentoses were tested with the purified enzyme as well as aldo- and ketohexoses, but none of these substances was active. It has been reported that the isomerase from *A. aerogenese* acts slowly on D-galactose to form a keto derivative (75). The preparation used, however, was a crude extract, and the specificity will have to be determined with a more highly purified preparation. The equilibrium of this isomerase is in favor of the aldopentose, as has been found with other aldopentose-ketopentose isomerases which have been investigated. About 14 per cent of the pentose exists in the keto form at 38° (34).

The phosphorylation of L-ribulose to L-ribulose 5-phosphate (reaction B, Fig. 1) is catalyzed by the enzyme ribulokinase. This enzyme has been purified about 120-fold from L. plantarum (12) and 200-fold from A. aero-

genes (76). Both enzymes show optimum activity at pH 7.0. They are inhibited by p-chloromercuribenzoate; and, glutathione (12, 75) or versene (75) is required during purification to retain activity. ATP and Mg++ are also required for the reaction. ITP was found to have some activity for the enzyme from A. aerobacter (75). One mole of ADP is formed per mole of L-ribulose utilized (12). Both enzyme preparations act on D- and L-ribulose, and the available evidence indicates that the same enzyme acts on both enantiomorphs (12). D- and L-xylulose and aldopentoses tested were not active. The enzyme from A. aerogenes, however, also phosphorylates adonitol and L-arabitol at appreciable rates (75). The reaction product formed from L-ribulose was purified by chromatography on Dowex-1-formate and isolated as the barium salt (12, 75). The pentose was identified as ribulose and distinguished from xylulose on the basis of the cysteine-carbazole test (12). The product was shown to be the L-enantiomorph on the basis of enzymic tests (12) and the position of the phosphate group was determined by measuring hydrolysis rates of the periodate reaction product (12, 75). Although the properties of the ribulokinase obtained from both organisms were similar, some differences were observed. The half-saturating concentration of L- and D-ribulose for the enzyme from A. aerobacter was about one fourth of that observed with the L. plantarum enzyme, although the K. values for ATP are about the same. The K, value for Mg++ is equal to the value found for ATP using the A. aerobacter enzyme, while the value for Mg++ is one half that of the ATP value using the enzyme from L. plantarum.

The epimerization of L-ribulose 5-phosphate at C-4 to form D-xylulose 5-phosphate (Reaction C, Fig. 1) was first demonstrated in extracts of A. aerogenes by Wolin, Simpson & Wood (99). This enzyme is also present in extracts of L. plantarum (11) and is distinct from the "3-epimerase" which catalyzes the reversible conversion of D-ribulose 5-phosphate to Dxylulose 5-phosphate (1, 84) (Reaction F, Fig. 1). The 4-epimerase has been purified about 200-fold from both organisms (13, 100). The assay methods for the 4-epimerase were based on the use of purified enzymes involved in the subsequent metabolism of p-xylulose 5-phosphate in the two fermentations. The product of the reaction was identified as p-xylulose-5-phosphate by measuring the oxidation of DPNH in an assay system containing transketolase and α-glycerol phosphate dehydrogenase (100). The UDPG 4epimerase described by Maxwell (51) requires DPN as a cofactor. No evidence was obtained for a cofactor requirement with either of the ribulose 4-epimerase preparations, and no DPN or DPNH could be detected in the purified enzyme from L. plantarum (13). The equilibrium ratio of p-vylulose phosphate to L-ribulose phosphate was 1.2 at 37° with the enzyme from L. plantarum (13), and 1.8 to 2.0 at 24° with the A. aerogenes enzyme. The equilibrium ratio of p-xylulose phosphate to p-ribulose phosphate in the reaction catalyzed by 3-epimerase was found to be 1.5 (32) and 1.4 (1).

p-Xylulose-5-phosphate occupies a singular position in pentose metabolism by microorganisms (see Fig. 1). It may be formed from p-ribose by

the action of ribokinase, phosphoriboisomerase, and 3-epimerase, and these enzymes have all been shown to occur in *L. plantarum*. Its formation from p-xylose by isomerization and phosphorylation has been demonstrated in a number of organisms. The enzymes responsible for its formation from L-arabinose have just been described and have been demonstrated in *A. aerogenes* and *L. plantarum*.

The subsequent metabolism of D-xylulose-5-phosphate by L. plantarum (11) and A. aerobacter (75) differs. D-xylulose-5-phosphate undergoes a phosphorolysis in the presence of an enzyme phosphoketolase present in L. plantarum to form acetyl phosphate and triose phosphate as shown by reaction D in Figure 1 (32). No evidence has been found for the metabolism of D-xylulose-5-phosphate via transketolase in this organism. The phosphoketolase has been purified from L. plantarum extracts about 44-fold. The minimum molecular weight was found to be about 550,000 by sedimentation analysis. Thiamine pyrophosphate, orthophosphate, Mg^{++} , and thiols are required for activity. The K_s for thiamine pyrophosphate is $2.1 \times 10^{-6} M$. Despite the high affinity of the coenzyme for phosphoketolase, the enzyme was resolved very early during the purification procedure. The reaction could not be demonstrated in the reverse direction. The purified enzyme was specific for D-xylulose-5-phosphate, and no activity was observed with fructose 6-phosphate.

An enzyme has been purified by Schramm, Klybas & Racker (72) from glucose-grown Acetobacter xylinum which catalyzes the phosphorolysis of fructose 6-phosphate to yield acetyl phosphate and erythrose 4-phosphate. This enzyme has been purified twentyfold. The partially purified preparation also catalyzes the phosphorolysis of xylulose-5-phosphate to yield acetyl phosphate. It has no activity on other substrates tested. The properties of the phosphoketolase from A. xylinum are similar to those of the enzyme derived from L. plantarum in certain respects, but differ significantly in a number of others. The phosphoketolase of A. xylinum does not require thiamine pyrophosphate for activity, but is stimulated by this cofactor in the presence of Mg⁺⁺. No thiol is required for activity of the enzyme. Both enzymes catalyze the cleavage of the ketose in the presence of arsenate in place of phosphate, to yield acetate rather than acetyl phosphate. Both enzymes have maximum activity near pH 6.0, and very little activity above pH 7.0.

The acetyl phosphate and triose phosphate formed from D-xylulose-5-phosphate by phosphoketolase is further metabolized by enzymes present in *L. plantarum*. Acetokinase which catalyzes the formation of acetate and ATP from acetyl phosphate and ADP (Reaction E, Fig. 1) has been found in these organisms; and the conversion of D-glyceraldehyde phosphate to lactic acid is presumed to occur via glycolysis (33) with the formation of two moles of ATP. The over-all fermentation of pentose by *L. plantarum*

resulting from enzymatic reactions shown in Figure 1 may be represented by Equation 1:

pentose
$$+ 2 \text{ Pi} + 2 \text{ ADP} \rightarrow \text{acetate} + \text{lactate} + 2 \text{ ATP}$$
 1.

and results in the net formation of two pyrophosphate bonds per mole of pentose fermented.

Phosphoketolase has also been demonstrated in glucose-grown Lactobacillus mesenteroides by Hurwitz (38). The products of the fermentation of glucose by this organism are equimolar quantities of CO₂, lactic acid, and ethanol. It has previously been shown by De Moss (20) that a ketopentose phosphate and CO₂ are formed from glucose 6-phosphate. These observations suggest that the cleavage of pentose by Lactobacillus plantarum and Leuconostoc mesenteroides occurs by a common mechanism, and that the ethanol and lactic acid formed as fermentation products by L. mesenteroides are derived from the acetyl phosphate and triose phosphate formed as products of the phosphoketolase catalyzed cleavage of pentose. Phosphoketolase occurs not only as an inducible enzyme in pentose fermentation, but is also required for the fermentation of hexoses in at least this one lactic acid organism.

An alternate pathway of metabolism of D-xylulose-5-phosphate and pentoses in A. aerogenes has been described by Simpson, Wolin & Wood (75). When extracts of this organism were incubated with L-ribulose, Mg++ and ATP, a number of carbohydrate derivatives, including glucose, sedoheptulose, fructose, ribose, ribulose, sedoheptulose phosphate, fructose phosphate, ribulose phosphate, and arabinose phosphate, were identified among the reaction products. It was therefore postulated that D-xylulose-5-phosphate is metabolized via transketolase and transaldolase to yield fructose-6-phosphate, which is then degraded by the glycolytic enzymes. However, the direct demonstration of the presence of transketolase and transaldolase and the absence of phosphoketolase in these extracts has not been reported. A specific DPNH-dependent D-ribulose dehydrogenase which converts ribulose to ribitol has been demonstrated in A. aerogenes (29, 101), and has been partially purified by Fromm (29).

Pentose metabolism by clostridia appears to be similar to that observed in A. aerogenes, rather than that observed with the lactic acid bacteria. Cynkin & Delwiche (17) and Cynkin & Gibbs (18) have studied the fermentation of D-ribose by Clostridium perfringens. D-Xylose and L-arabinose are not fermented by this organism, even after induction periods. The occurrence of ribokinase activity was inferred from the observation that D-ribose is metabolized when incubated with cell extracts in the presence of ATP. The presence of a phosphopentoisomerase was inferred from the formation of cysteine-carbazole reactive material, presumed to be ketopentoses, from ribose-5-phosphate. Pentose phosphate was converted to hexose phosphate in the presence of the extract. Of the 15 µM of pentose utilized, 3 µM were

recovered as hexose. It is possible that most of the hexose formed in the postulated reactions is metabolized to other end products. It is also possible, however, that enzymes other than transketolase and transaldolase are responsible for the observed pentose metabolism. The metabolism of pentoses via the postulated scheme was confirmed, however, by tracer studies with pentose-1-C¹⁴. The specific activity of the CO₂, acetate, and ethanol formed agreed well with a fermentation mechanism in which fructose-6-phosphate is formed by the action of transketolase and transaldolase and is further metabolized to yield CO₂, ethanol, and acetate by the glycolytic pathway. The results obtained in the fermentation of glucose-2-C¹⁴ are consistent with this assumption, and are in disagreement with the previous results of Paege, Gibbs & Bard (56). The metabolism of hexoses by Clostridium oedematiens via the glycolytic enzymes has been inferred from the observation that fructose-1,6-diphosphate aldolase occurs in the organism (74).

The fermentation of deoxyribose and its derivatives has been reported in a number of systems. The deoxyribose moiety of thymidine is converted to equimolar quantities of ethanol, formate (or CO_2 and H_2), and acetate by anaerobically grown cells of $E.\ coli\ (35)$. This fermentation has been adapted to the total degradation of deoxyribose for isotopic studies by Bernstein, Fossitt & Sweet (10). It has been shown (10) that the deoxyribose moiety of thymidine is split as shown in Figure 2, and that there is negligible mixing of the carbon atoms.

Fig. 2

2-Deoxy-D-ribose was made available by a simplified chemical synthesis described by Diehl & Fletcher (21), and fermentation of this substrate by L. plantarum was studied by Domagk & Horecker (22). Cells grown on other pentoses do not ferment 2-deoxy-D-ribose. When the fermentation is carried out in the presence of bisulfite, equivalent amounts of lactic acid and acetaldehyde are formed. In the absence of bisulfite, ethanol and acetic acid are also found, and are presumed to arise by a dismutation of acetaldehyde. The presence of deoxyribose aldolase was demonstrated in cell extracts, and the enzyme was partially purified. This enzyme, first demonstrated by Racker (60) in E. coli, catalyzes the reversible cleavage of 2-deoxy-D-ribose-5-phosphate to acetaldehyde and glyceraldehyde-3-phosphate. Evidence has also been obtained for the presence of a deoxyribokinase.

Deoxyribose is presumably fermented by E. coli in an analogous manner,

and the acetaldehyde formed is reduced to ethanol while the glyceraldehyde phosphate is oxidized to pyruvate, which is converted to formic acid and acetic acid.

Uronic Acids.—The enzymatic reactions involved in the bacterial metabolism of the uronic acids (glucuronic and galacturonic, see Figure 3) have been reported from several laboratories during the past year. A number of bacterial species have been employed for this work, including available laboratory strains of E. coli (92, 95), Erwinia (83) and strains of Escherichia, Klebsiella, Alcaligenes (19), Serratia (57), and Aerobacter (52) obtained by the enrichment culture technique on media containing uronic acids or 5-ketogluconic acid (19) as the carbon source. The metabolism of uronates has been found to involve an inducible system in all these organisms.

Watanabe & Arai (95) investigated the metabolism of D-galacturonic acid by E. coli (K-12), and detected D-galacturonic acid and 5-keto-L-galactonic acid as reaction products. ATP is required for the degradation of D-glucuronic acid by extracts of acetone powders of the organism. On the basis of these observations, the authors proposed that D-glucuronic acid is metabolized by the following sequence of reactions [also see Figure 3]:

glucuronic acid → galacturonic acid → 5-keto-galactonic acid → 5-keto-6-phosphogalactonic acid.

The reactions of this postulated scheme involve inversion of the configuration at C-4 of glucuronic acid, isomerization of the alduronic acid to the ketouronic acid, and phosphorylation of the ketouronic acid. These reactions, as well as the related reactions involved in the metabolism of galacturonic acid, hae subsequently been demonstrated by other workers using different bacterial species which metabolize the uronic acids.

Wahba, Hickman & Ashwell (92) demonstrated and purified a uronic acid isolmerase from extracts of *E. coli* grown on D-galacturonic acid. A partially purified extract of these organisms isomerized the aldouronic acid, D-galacturonic acid, to the keto uronic acid, D-tagaturonic acid, and D-glucuronic acid to D-fructuronic acid. The products were isolated by chromatography on anion exchange resin or by paper chromatography. The reversibility of the reactions was also demonstrated. Tagaturonic and fructuronic acids, as indicated in Figure 3, may also be regarded as 5-ketoaldonic acids. Although 5-ketogluconic acid has been encountered as a product of glucose metabolism by *Acetobacter* species (66), the enzymes involved in its formation and subsequent metabolism were not known, and the metabolic significance of this class of compounds in other bacterial species had not been previously appreciated.

The metabolism of 5-ketogluconic acid has been investigated by de Ley (19), using strains of *Escherichia*, *Klebsiella*, and *Alcaligenes* isolated from soil and water on media enriched with 5-ketogluconic acid. The oxidation of 5-ketogluconic acid was inducible in the organisms tested. Cells grown on gluconic acid or 5-ketogluconic acid, however, oxidized gluconic acid without

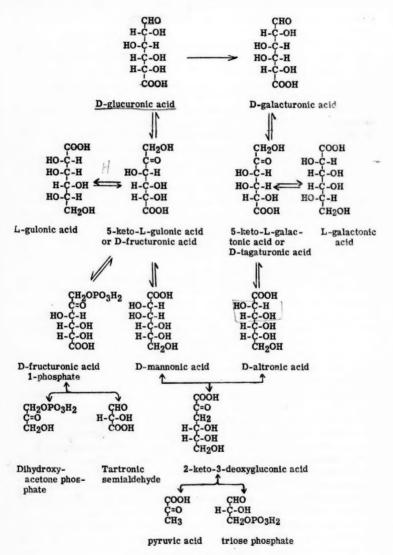


Fig. 3. Uronic acid metabolism.

any induction period. This suggested that 5-ketogluconic acid is metabolized via a reduction to gluconic acid:

5-ketogluconic acid + DPNH + H+

gluconic acid + DPN+.

A reversible reduction of 5-ketogluconic acid to gluconic acid with DPNH or TPNH was shown, using cell extracts of these organisms. It was further shown that a 5-ketogluconic acid kinase was present in *Klebsiella* and caused the disappearance of 5-ketogluconic acid in the presence of ATP. The product, presumed to be 5-keto-6-phosphogluconate, was not demonstrated. The relation of this enzyme to the p-tagaturonic acid kinase of Watanabe & Arai (95) remains to be determined.

Starr and his co-workers (83) found that the metabolism of D-galacturonic acid and D-glucuronic acid by cell extracts of *Erwinia* and *Aerobacter* grown on either substrate was accompanied by the oxidation of DPNH and TPNH. The products of the reaction were identified as L-galactonic acid and L-gulonic acid, respectively (see Fig. 3). Later experiments by Kilgore & Starr (44) demonstrated that the over-all reaction involves an initial isomerization of the galacturonic acid to 5-keto-L-galactonate followed by an enzymic reduction of the keturonic acid to the aldonic acid. These two enzymes have been separated and partially purified from extracts of *Erwinia* grown on galacturonic or glucuronic acid. The reductase reaction is faster with DPNH than with TPNH. The enzyme does not act on D-galacturonate, 5-keto-D-gluconate, 2-keto-D-gluconate, or 2-keto-L-gulonate; however, 5-keto-L-gulonate was not available for testing.

Payne & McRorie (57) have shown that cell extracts of *Serratia* grown on D-glucuronic acid catalyze the isomerization of D-glucuronic acid to D-fructuronic acid and the reduction of this ketouronic acid to L-gulonic acid in the presence of TPNH. Galacturonic acid is metabolized by the same extracts. The products of this reaction, however, have not been identified. Since a crude cell extract was employed, it was not possible to determine

the specificity of this enzyme.

Ashwell, Wahbe & Hickman (1a) purified an enzyme from cells of *E. coli* grown on D-galacturonic or D-glucuronic acid which catalyzes a reversible reaction in which D-tagaturonic acid or D-fructuronic acid is reduced in the presence of DPNH to D-altronic or D-mannonic acid, respectively (Fig. 3). These are the epimers of the reduction products L-galactonic and L-gulonic acids identified as reaction products formed by reduction of the corresponding keto uronic acids in experiments described by other workers (57, 83). Ashwell, and his co-workers (1a), showed that the reaction was reversible, but that L-galactonic acid and L-gulonic acid are completely inert with the purified enzymes.

2-Keto-3-deoxygluconic acid was identified as a common intermediate formed from both D-altronic and D-mannonic acids. This product is eventually cleaved in the presence of ATP to form pyruvic acid and glyceraldehyde

3-phosphate (Fig. 3).

An alternate mechanism of ketouronic acid metabolism suggested by Watanabe & Arai (95) and by de Ley (19), involves an enzymic phosphorylation of p-tagaturonic acid (95) or 5-ketogluconic acid (19) with ATP. McRorie & Novelli (52), in studying the anaerobic metabolism of glucuronic acid by Aerobacter aerogenes, found that there were approximately two moles of CO2, two moles of volatile acid and two moles of H2 formed per mole of substrate utilized. Aerobically adapted cells formed volatile acids, but failed to form CO₂ and H₂. This suggests that the metabolism of glucuronic acid by this organism does not occur by the decarboxylation of gulonic acid formed by the isomerase and dehydrogenase. Evidence was obtained for an aldolasetype cleavage of fructuronate by extracts of cells grown anaerobically on glucose or glucuronate. ATP and Mg++ are required for the reaction, suggesting that the substrate of the aldolase-type reaction is a phosphorylated derivative of fructuronic acid, probably fructuronate-1-phosphate. The formation of D-fructuronic acid from dihydroxyacetone phosphate and tartronic semialdehyde by the extracts, as shown in Figure 3, was suggested by the formation of a compound reacting in the cysteine-carbazole test. The products of the reaction are further metabolized by anaerobically adapted cells to yield acetic acid, H2, and CO2. It was suggested that dihydroxyacetone phosphate is converted to pyruvic acid by the normal glycolytic enzymes with the reduction of DPN to DPNH. The reduced pyridine nucleotide is then reoxidized in a coupled reduction of tartronic semialdehyde to glyceric acid. The occurrence of a tartronic acid semialdehyde dehydrogenase in glucuronate-grown cells was demonstrated by measuring the oxidation of DPNH in the presence of this substrate. Only very low levels of this enzyme could be demonstrated in glucose-grown cells. Although the product of the reaction was not characterized, it was found that glyceric acid and 3-phosphoglyceric acid are metabolized to H₂ and CO₂ by these extracts. The purification of the enzymes and the isolation of the intermediates proposed would further support this pathway of ketouronic acid metabolism. Future studies should clarify the relation between this pathway of uronic acid metabolism in Aerobacter and that demonstrated by Ashwell and coworkers (1a) in E. coli.

NITROGENOUS COMPOUNDS

Glutamic acid and histidine.—The fermentation of amino acids by Clostridium tetanomorphum has recently been reviewed by Barker (4). This organism ferments a number of amino acids, but histidine and glutamic acid are fermented with particular facility. The initial steps in the fermentation of histidine by C. tetanomorphum are probably similar to those demonstrated with Pseudomonas fluorescens (88), Aerobacter aerogenes (49), and animal tissues (89). Histidine is deaminated to urocanic acid and the imidazole moiety is then hydrolytically cleaved to yield formiminoglutamic acid (see Fig. 5). The products formed from the formimino moiety of formiminoglutamic acid, however, vary in the different systems studied. Formic acid, NH₃, and glutamic acid are formed by Pseudomonas extracts, and formyl-

glycine has been identified as an intermediate. Formic acid and NH_3 are also formed by mammalian liver preparations; however, formylglutamic acid is not an intermediate, and it has been shown that the formimino group is enzymically transferred as a unit and is subsequently metabolized to form formic acid and ammonia (90). In the case of A. aerogenes and C. tetanomorphum, formiminoglutamic acid is metabolized to yield formamide and glutamic acid. Although this evidence suggests that formiminoglutamic acid is metabolized by three different pathways by these different organisms, the enzymes involved in the bacterial reactions have not yet been purified. Glutamic acid, however, is formed from histidine in all these systems.

The conversion of urocanic acid to formininoglutamic acid is presumed to occur via 4-imidazolone-5-propionic acid as an intermediate (Fig. 4).

Fig. 4. Histidine metabolism.

Evidence for the formation of a compound with the spectroscopic and chromatographic properties of this postulated compound has been obtained, using extracts of A. aerogenes, by Revel & Magasanik (68). The compound is enzymically converted to glutamic acid and formamide. Like other 4-imidazolones unsubstituted in the 2-position (28), however, the intermediate is unstable and forms a mixture of DL- and L-formylisoglutamine by non-enzymic reactions. Similar results were obtained by Feinberg & Greenberg (25), using a preparation of urocanase from mammalian sources.

The degradation of ergothionine by a strain of Alcaligenes faecalis, isolated by enrichment culture techniques from soil by Yanasugondha & Appleman (104), is analogous to the degradation of histidine catalyzed by histidase. The products of the reaction are 2-thiolurocanic acid and trimethylamine. The trimethylamine is not further metabolized. The thiolurocanic acid, however, is slowly metabolized. If the reactions involved in the metabolism of this substance are analogous to those postulated in urocanic

acid metabolism, one would expect 2-thiol-4-imidazolone propionic acid as an intermediate. This compound, in contrast to the intermediate derived from histidine, would be expected to be stable.

The products of the fermentation of glutamic acid by C. tetanomorphum are butyric and acetic acids, carbon dioxide, ammonia, and hydrogen (4). Further studies on this fermentation by Barker and his colleagues have revealed that the glutamic acid is metabolized in a unique manner, only one mole of CO_2 being evolved per mole of glutamic acid fermented. The fermentation therefore does not involve α -ketoglutaric and the tricarboxylic acid cycle.

The formation of the branched chain dicarboxylic acid, mesaconic acid, from glutamic acid has been studied, using extracts of *C. tetanomorphum* (54)

Fig. 5. Glutamic acid metabolism by Clostridium tetanomorphum.

(see Fig. 5). Glutamic acid-4-C¹⁴ is converted to mesaconic acid which contains the C¹⁴ exclusively in the carbon adjacent to the methyl group. In order to account for this labeling pattern, as well as results obtained in other tracer studies, it is necessary to assume that carbon atoms 1 and 2 are transferred as a unit and form a new bond with C-4 of the glutamic acid.

Several of the enzymes involved in the interconversion of glutamic acid and mesaconic acid have been purified. The product formed from mesaconic acid and ammonia by charcoal-treated cell extracts has been identified as o-L-threo- β -methylaspartic acid (8). The enzyme which catalyzes this reaction, β -methylaspartase, has been purified about 45-fold (5, 7). The purified enzyme has a specific requirement for Mg $^{++}$ and a nonspecific requirement for a monovalent cation. The enzyme acts on L-threo- β -methylaspartic acid, L-erythro- β -methylaspartic acid and L-aspartic acid. Neither the D-threo or

D-erythro isomers of β -methylaspartic acid are active. The maximum reaction velocity is obtained with L-threo- β -methyl aspartic acid. The reaction is reversible, and both fumaric acid and mesaconic acid are active. The equilibrium for the reaction in the direction of mesaconic acid formation at pH 9.7 is 0.306.

The conversion of glutamic acid to β -methylaspartic acid requires the addition of a cofactor present in boiled cell extracts. The cofactor has been purified from C. tetanomorphum and identified as a derivative of pseudovitamin B_{12} (6). Pseudovitamin B_{12} contains adenine in place of dimethylbenzimidazole. This form of the coenzyme has been called adenine- B_{12} coenzyme. In addition to this compound, the benzimidazole- and the dimethylbenzimidazole- B_{12} coenzymes have been prepared by growing C. tetanomorphum in the presence of the respective heterocyclic bases, and the coenzymes have been isolated by the use of ion exchange chromatography (96).

All three coenzymes differ from the corresponding vitamins in containing a mole of adenine in addition to the heterocyclic base. All three forms of the coenzymes are active in the enzyme assay. The relative molar activities of the benzimidazole-, adenine-, and dimethyl benzimidazole- B_{12} coenzymes are 100, 17, and 1.6, respectively. The coenzymes are sensitive to treatment with light and cyanide. Treatment of the coenzymes with cyanide results in the liberation of one mole of adenine and the formation of the corresponding form of vitamin B_{12} . The coenzyme contains cobalt, and the available evidence indicates that the coenzyme is composed of a vitamin B_{12} moiety with an adenine residue probably linked to a double bond in the corphyrin ring system.

The dimethylbenzimidazole- B_{12} coenzyme has also been obtained from rabbit liver and dried cells of *Propionibacterium*. It seems probable that the coenzymes are the predominant naturally-occurring forms of vitamin B_{12} . The failure to detect them previously is possibly related to the light and

cyanide sensitivity of the coenzymes.

The manner in which the coenzyme participates in the conversion of glutamic acid to mesaconic acid is intriguing. The relation of this reaction to others in which vitamin B_{12} participates, such as the synthesis of methio-

nine, deoxyribonucleosides, and protein, is also of great interest.

Glutamine.—A unique energy-yielding reaction has been suggested by the work of Smith on the nutritional requirements of pleuropneumonia like organisms. These organisms cannot metabolize hexoses (77, 78); however, they can be grown under anaerobic conditions in a synthetic medium containing amino acids. Glutamine is required for growth by all strains tested, and it is degraded to glutamic acid (79). Cell extracts also degrade glutamine in the presence of ADP, Mg⁺⁺, and orthophosphate at pH 6.0 to 6.5, and the ADP is converted to ATP. It is presumed that the phosphorolysis of glutamine (Reaction 2) is the energy-yielding reaction of this fermentation:

Growth of the organisms on glutamine, however, has not been demonstrated. At pH 8.0, the deamination of glutamine occurs in the absence of ADP, presumably by a hydrolytic reaction. The hydrolase shows optimal activity at pH 8.0, which is also the optimum pH for growth of the organism.

Glycine.—The reduction of glycine to yield acetic acid and ammonia (Reaction 3) occurs in a variety of organisms in a fermentation coupled to the oxidation of other amino acids:

$$H_2N - CH_2 - COOH + 2H \rightarrow CH_3COOH + NH_3.$$
 3.

Clostridium stricklandii, which has been shown by Stadtman & White (80) to carry out the fermentation of ornithine coupled to the reduction of proline or lysine, has been used as a source of an extract which will catalyze the reductive deamination of glycine according to Reaction 3 in the presence of dithiols such as 1,3-dithiolpropanol (81). The 1 and 2 carbon atoms of glycine appear as the 1 and 2 carbons, respectively, of acetic acid and do not undergo mixing in this reaction. Partially purified preparations require the addition of ADP and orthophosphate. The stoichiometry for the following reaction was established by Stadtman et al. (82):

$$H_2N$$
— CH_2 — $COOH + Pi + ADP + R(SH)_2 \rightarrow CH_3COOH + NH_2 + ATP + RSS. 4.$

This represents another reaction in which ATP can be generated from ADP and orthophosphate, and is apparently unique in being coupled to an over-all reductive reaction. The enzyme preparation has been partially purified, and a number of cofactors including DPN+, pyridoxal phosphate, and Mg++ have been found to stimulate the reaction under some conditions. Dimercaptans are the only active electron donors. The enzyme preparation has been separated into two fractions involved in the formation of ATP and acetate. However, the stoichiometry shown by Reaction 4 is no longer obtained with the purified fractions. Instead, it is found that over four moles of orthophosphate are esterified per mole of acetate formed. This suggests that a phosphorylated intermediate is formed under these conditions. When glycine-C¹ is used as a substrate with the purified enzyme fractions, a volatile, radioactive product distinct from acetate or glycine is detected, which is presumed to be the postulated intermediate. However, the compound has not yet been identified.

The fermentation of glycine by the anaerobes, Diplococcus glycinophilus and Clostridium acidi-urici, probably occurs by another pathway, since the fermentation of glycine 2-C14 by these organisms yields acetate labeled in both positions (61, 70). The fermentation of glycine to acetate and CO2 by D. glycinophilis is an energy-yielding process, since this organism can use glycine as its sole source of C, N, and energy for growth. Pyruvic acid is probably the key intermediate in the fermentation of glycine by D. glycinophilus, and it has been proposed by Sagers & Gunsalus (70), on the basis of tracer experiments and enzyme analysis, that the fermentation proceeds by a pathway involving the following series of reactions:

It seems doubtful that these reactions relate to the oxidative fermentation of glycine seen by Campbell (15) and Bachrach (3) with *Pseudomonas* strains. In this fermentation glyoxylic acid has been implicated as an intermediate, since the formic acid and carbon dioxide formed in the oxidative process are derived from the carboxyl and methylene carbons of glycine, respectively. In the *D. glycinophilus* fermentation the C₁-fragment (which is equivalent to formic acid) and the carbon dioxide formed in Equation 5, are derived from the methylene and carboxyl carbons, respectively, of glycine while the carbon dioxide formed in Equation 8 is also derived from the carboxyl carbon of glycine.

Threonine and serine.—Serine and threonine are fermented by a Gramnegative coccus isolated from rumen contents to yield acetic acid and propionic acid, respectively [Lewis & Elsden (48)]. The fermentation of lactic acid and acrylic acid by this organism has also been described in preliminary reports by Ladd & Walker (45, 93). Cells of this organism grown on lactic acid and corn-steep liquor were used for the purification of L-threonine deaminase. The enzyme was purified about 100-fold by Walker (94). 2-Oxybutyric acid, which is an intermediate in the fermentation of threonine, has been identified as the product of the enzymatic reaction. This bacterial threonine deaminase is believed to be active in the deamination of serine to yield pyruvic acid, in this respect resembling the purified threonine deaminase of E. coli and Neurospora, and differs from the mammalian enzyme which is specific for threonine. The bacterial enzyme requires the addition of pyridoxal phosphate for activity after lyophilization in the presence of ammonium sulfate.

Creatinine.—The fermentation of creatinine by an organism tentatively identified as Clostridium paraputrificum, has been described by Szulmajster (86). In addition to creatinine, the organism utilizes carbohydrates, creatine, and guanidoacetic acid for growth. The utilization of creatinine is inducible. The fermentation of creatinine results in the formation of an equivalent amount of ammonia and about 0.80 of an equivalent of N-methylhydantoin. Small amounts of volatile acids were also detected. Since the organism grows on creatinine and accumulates N-methylhydantoin, it was suggested that the conversion of creatinine to N-methylhydantoin is the energy-yielding reaction in this fermentation (Fig. 6). Since only 75 to 80 per cent of the creatinine fermented is recovered as methylhydantoin, however, it is possible that the energy for growth is derived from reactions involved in the conversion of methylhydantoin to volatile acids.

Some support for the theory that the conversion of creatinine to methylhydantoin is an energy-yielding reaction was obtained in studies with dialyzed cell extracts (87). Such preparations require the addition of ATP or ADP and orthophosphate for optimum utilization of creatinine. Creatine could not be substituted for the creatinine. The reaction with ADP resulted in the formation of ATP. However, the amount of ATP formed was much less than the amount of creatinine utilized. The enzyme catalyzing this reaction, creatinine desimidase, was purified about thirtyfold by treatment with protamine and ammonium sulfate. It was then chromatographed on N,N-diethylaminoethylcellulose, and three major components were obtained which accounted for all the original enzyme activity. One of these fractions, representing 25 per cent of the initial activity, was purified about 140-fold by this procedure. This represents a 4300-fold purification. The activity of the purified enzyme was not affected by adenylic acid nucleotides, in contrast

HN=C
$$\stackrel{NH-C=O}{\longrightarrow}$$
 O=C $\stackrel{N-C=O}{\longrightarrow}$ + NH₃

Creatinine N-methyl hydantoin

Fig. 6

to the crude preparation. The purified enzyme was stimulated by the addition of orthophosphate; however, this was a nonspecific effect which was also observed upon the addition of polyvalent anions, such as versene and polyphosphate.

Purines.—The fermentation of purines by clostridia offers another example in which the main energy-yielding reaction is probably one other than the oxidation of pyruvic acid. Clostridium cylindrosporum ferments guanine with the formation of carbon dioxide and roughly equivalent amounts of formic acid and glycine (62). A small amount of acetic acid is also formed. The degradation of xanthine by cell extracts proceeds, by a series of hydrolytic reactions, to yield formiminoglycine (63). The degradation of formiminoglycine to formic acid and glycine requires ADP and orthophosphate, as shown by Reaction 9, and results in the formation of ATP (64):

HOOC—
$$CH_2$$
— NH — CH = NH + ADP + Pi
 \rightarrow HOOC— CH_2 — NH_2 + HCOOH + NH_3 + $ATP.$ 9.

This over-all reaction has been found to result from the action of at least four enzymic reactions. One of these, represented by Equation 10, results in the formation of ATP from ADP and orthophosphate:

10-formyl-tetrahydrofolic acid + ADP + Pi

≓ HCOOH + tetrahydrofolic acid + ATP. 10.

The enzyme catalyzing this reaction is known as tetrahydrofolic formylase, and it has been isolated in crystalline form from C. cylindrosporum (65). This enzyme constitutes roughly three per cent of the dry weight of the organism.

Acetic acid and carbon dioxide are formed in the fermentation of guanine by the closely related organism Clostridium acidi-urici; but, in contrast to the purine fermentation carried out by C. cylindrosporum, no significant amounts of glycine or formic acid are detectable. Formiminoglycine is also formed from xanthine by extracts of this organism. The organism contains high concentrations of tetrahydrofolic formylase. Formiminoglycine, however, is metabolized by suspensions of this organism to form acetic acid and carbon dioxide (61, 63). The results of experiments with C¹⁴-labeled substrates are consistent with a pathway involving the conversion of formiminoglycine to glycine and an active C₁-moiety, probably 10-formyltetrahydroglycine to glycine and an active C som serine, as shown in Reaction 6, and is further metabolized via pyruvic acid to yield acetate, as in Reactions 7 and 8. The enzymes catalyzing these reactions have been demonstrated in extracts of C. acidi-urici (59, 70).

The fermentation of purines by Micrococcus lactilyticus (97) and Micrococcus aerogenes (98) has been described by Whiteley. The formation of urea (97), pyrimidines, and lactic and propionic acids (98) in these fermentations suggests that the pathway differs from that demonstrated in the clostridia.

The fermentation of purines by washed cell suspensions of organisms present in cow rumen has been observed by Jurtshuk, Doetsch & Shaw (43). Acetic acid was identified as a fermentation product, but higher volatile acids and lactic acid could not be detected. Uric acid, xanthine, and guanine were readily fermented by washed cell suspensions, whereas adenine and hypoxanthine were not. Both these observations suggest that the purine is degraded by a process related to the fermentation carried out by the clostridia rather than by the *Micrococcus*. This is unexpected since the rumen normally contains relatively large numbers of micrococci and only small numbers of clostridia.

The aerobic degradation of uric acid has been studied with a *Pseudomonas* strain isolated by enrichment culture techniques by Bachrach (2), and by a parasitic strain of *Microsporum* (85). The degradation probably proceeds via allantoin, allantoic acid, and glyoxylic acid with both organisms. This was shown with the Pseudomonad, using the sequential induction technique (2), and by demonstrating the formation of the intermediates by the *Microsporum* (85).

Pyridine derivatives .- An anaerobic, spore-forming organism which

utilizes nicotinic acid as a source of carbon, nitrogen, and energy for growth was isolated by Harary (30) from mud, using the enrichment technique. The organism also grows on glucose, pyruvate, and nicotinamide, but not on pyridine. The fermentation is described by Equation 11:

$$C_6H_5NO_2 + 4H_2O \rightarrow NH_3 + CO_2 + CH_3COOH + CH_3CH_2COOH.$$
 11.

Nicotinic acid is reversibly oxidized to 6-hydroxynicotinic acid by a suspension of lyophilized cells incubated in the presence of methylene blue under anaerobic conditions (31) (Equation 12). Since the reaction occurs under anaerobic conditions, it may be assumed that the oxygen of the hydroxyl group is derived from water, and not from O₂.

$$H_2O$$
 + nicotinic acid \rightarrow 6-hydroxynicotinic acid + 2H. 12.

The degradation of nicotinic acid by bacteria under aerobic conditions has been investigated by several workers (9, 16, 26, 36, 39, 91). It is of interest that the first step in the aerobic degradation of nicotinic acid also involves hydroxylation at the 6-position (36). It was originally thought that the source of the oxygen atom of the hydroxyl group was oxygen gas. It has recently been shown by Hunt, Hughes & Lowenstein (37), however, that the source is $\rm H_2O$ in the aerobic organism.

The intermediates in the degradation of 6-hydroxynicotinic acid have been demonstrated by Behrman & Stanier (9), using extracts of a Pseudomonas isolated by enrichment techniques. The reaction sequence deduced from these results involves the oxidative decarboxylation of 6-hydroxynicotinic acid to 2,5-dihydroxynicotine, and oxidative cleavage of the dihydroxy-compound to formic and fumaric acids. Pyruvic acid has been identified as an intermediate, suggesting that fumaric acid is metabolized by the known enzymes to form this product. It is possible that the anaerobic fermentation described by Harary (30, 31) follows a similar pathway and that the energy for growth is derived from reactions associated with the conversion of formic and fumaric acids to the observed fermentation products CO₂, acetate, and propionate. However, it seems doubtful that the process of oxidative aromatic ring cleavage which has commonly been observed among the aerobic bacteria can operate in anaerobic metabolism, since this process involves the addition of 2 atoms of oxygen derived from oxygen gas (50). It would, therefore, be extremely interesting to determine the metabolic reactions involved in the further degradation of 6-hydroxynicotinic acid by the anaerobic organism.

OXIDATIVE FERMENTATIONS

The degradations of a variety of organic compounds under aerobic conditions by organisms isolated from enrichment cultures, have been described during the past year. Studies with these organisms provide information on the metabolism of a variety of organic residues, such as acetylene

derivatives and heterocylic derivatives. In addition, a number of useful enzymes have been purified from the isolates.

An aldehyde dehydrogenase has been purified by Jakoby (41) from a strain of *Pseudomonas fluorescens* isolated from an enrichment medium containing ethylene glycol. The enzyme oxidizes aliphatic and aromatic aldehydes to the corresponding acids in the presence of pyridine nucleotides. Xanthine is not oxidized, and glyceraldehyde is the most readily oxidized substrate of those tested. The enzyme requires the addition of a thiol for activity. Dithiols are active at lower concentrations than monothiols. The enzyme is arsenite sensitive, and remains sensitive to this reagent even when lipoic acid is removed by specific enzymatic methods. However, the enzyme is not inhibited by arsenite in the absence of added monothiols, and it is suggested that the arsenite sensitivity reflects the presence of closely linked—SH groups at the active site of the enzyme (42).

An enzyme catalyzing the conversion of acetylene dicarboxylic acid to pyruvic acid and carbon dioxide has been purified by Yamada & Jakoby (102, 103) from a *Pseudomonas*. A facultative anaerobe which uses acetylene dicarboxylic acid as a C-source had been isolated by Eimhjellen (24); resting cells of this organism convert acetylene dicarboxylic acid to oxalacetic acid, as shown in Equation 13. Oxalacetic acid, however, is not an intermediate in the formation of pyruvate from acetylene dicarboxylic acid by this *Pseudomonas* (103).

$$\begin{array}{ccc} \operatorname{COOH} & & & & & & \\ | & & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ | & & & \\ |$$

The metabolism of various isomers of nitrobenzoic acid has been investigated by Durham (23) and Cain (14), using organisms isolated by enrichment culture on these substrates. The nitro group of these compounds was first reduced to the amino group, a reaction previously observed by Saz & Marmur (71) with E. coli extracts. The pathway of p-nitrobenzoic acid metabolism, indicated by sequential induction experiments, was through the corresponding amino- and hydroxy- derivatives to protocatechuic acid.

A strain of *Pseudomonas aeruginosa* was isolated from soil by Noe & Nickerson (55), using enrichment culture techniques with 2-pyrrolidone as a C- and N-source (Fig. 7). Cells grown on this substrate could utilize γ -aminobutyric acid without a lag period, and a specific γ -aminobutyric-glutamic acid transaminase was demonstrated in cell extracts. It was therefore suggested that the metabolism of 2-pyrrolidone by this organism proceeds by hydrolysis to γ -aminobutyric acid; this compound then undergoes a transamination reaction with α -ketoglutaric acid to yield glutamic acid

Fig. 7

and succinaldehyde. A γ -aminobutyric acid-glutamic acid transaminase has been purified by Scott & Jakoby (73) from a strain of *Pseudomonas* isolated by enrichment techniques from a medium containing pyrrolidine as the sole C-source. Since this organism does not grow on 2-pyrrolidone, it was suggested that 1,2-pyrroline, rather than the keto derivative, is an intermediate in the conversion of pyrrolidine to γ -aminobutyric acid.

The metabolism of indoleacetic acid by a *Pseudomonas* has been investigated by Proctor (58). The evidence obtained, using the sequential induction technique, indicated that indoleacetic acid is metabolized via skatole, indoxyl, salicylic acid, and catechole, and that indole, anthranilic acid, and o-aminophenol are not intermediates.

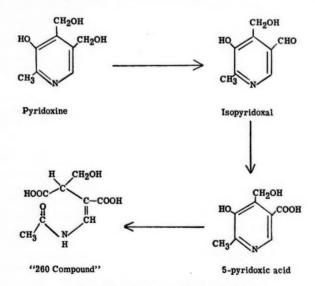


Fig. 8. Oxidative metabolism of pyridoxal.

The degradation of pyridoxine was investigated by Rodwell and his colleagues (69) and Ikawa et al. (40) with organisms isolated from soil. Isopyridoxal, 5-pyridoxic acid, and α -hydroxymethyl- α -(N-acetylaminomethylene)-succinic acid, or "260 compound," were identified as reaction products. The oxidation state of the compounds and the time course of their formation suggested a reaction sequence as shown in Figure 8. The main metabolic product of pyridoxine observed in mammalian species was 4-pyridoxic acid, but this compound was not formed in the bacterial degradation. The reaction involved in the splitting of the heterocyclic ring of pyridoxine involves the oxidation of a monohydroxy derivative to an unsaturated ketocarboxylic acid, whereas the splitting of other pyridine and aromatic derivatives by Pseudomonas species generally involves conversion of the compound to a dihydroxy derivative, which is then oxidatively split to form an unsaturated dicarboxylic acid (9).

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BACTERIAL GENETICS (WITH PARTICULAR REFERENCE TO GENETIC TRANSFER)^{1,2}

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This review is focused upon genetic transfer in bacteria and, in particular, those aspects which have recently received concerted attention.2 No attempt will be made to develop the topic from a historical perspective or to cover exhaustively any but the most recent literature. For the older literature, the reader is referred to other recent reviews of this series (61, 303) and elsewhere (147, 149). Consequences of genetic transfer mechanisms for interpretations of gene structure and function will be alluded to but briefly here; further discussion of this topic may be found in another review in this volume (72). Homologies between some phage and bacterial genes, apparent from completely independent lines of reasoning [contrast Stent, (337) with Luria et al. (267)], suggest common features in all phage-mediated genetic transfer, including transduction, conversion, and lysogenization; familiarity with lysogeny is essential to an understanding of phage-mediated genetic transfer. The reader is referred elsewhere for reviews of problems concerning phages and prophages (2, 43, 265, 268, 269); here we will restrict our discussion only to those very recent studies which have direct bearing on phagemediated transfer of "bacterial" traits.

All processes of recombination in bacteria so far extensively studied have revealed several common features: (a) the transfer of the genetic material is unidirectional, in each case involving donor bacteria and recipient bacteria; (b) only a portion of a single (haploid) complement is usually transferred; (c) DNA is implicated as underlying the genetic specificity of the transferred material; (d) the genetic fragments may be carried by the recipient bacteria in a variety of manners: (i) functional, nonreplicating;

¹ The survey of fiterature pertaining to this review was concluded in February, 1959.

² The following abbreviations will be used: DNA (deoxyribonucleic acid); HFT (high frequency transducing); Hfr (high frequency recombination).

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(ii) functional, replicating, but not stabilized; (iii) functional, attached to (or synapsed with) a specific site or sites, replicating in synchrony with the bacterial genome; or (iv) integrated into the bacterial genome as replacement for homologous genetic material.

Although the processes share many points in common, they are conveniently divided on the basis of the purely mechanical features of the transfer: (a) free DNA (transformation); (b) phage-mediated (transduction and conversion); and (c) cellular contact (conjugation). An additional section deals with bacteriocins and bacteriocinogenesis.

TRANSFORMATION

The rate in which interest in bacterial transformation is developing is indicated by the number of reviews on the subject currently available (79, 80, 165, 166, 167, 262, 323, 386). Particular attention is directed to the recent reviews by Ravin (303) and by Thomas (350).

A number of formal schemes have been proposed for the series of events leading to the production of the transformed cell (79, 80, 165, 166, 323). In general, these events include the following operational procedures: (a) the extraction of native DNA from the donor bacteria; (b) the uptake of this DNA by the competent cell, which presupposes two conditions: (i) the environmental conditions under which the DNA is taken up, and (ii) the development of cells which have the capacity to take up the DNA (competence); (c) the expression of transformed characteristics which may or may not be associated with (d) the incorporation or integration of genetic factors into the host cell; and (e) segregation of the recombinant genomes from the unaltered recipient genomes.

Limitations on any one of these processes could lead to the inability of a cell to undergo a transformation. It is, therefore, not surprising that there have been a number of reports of transformations which have been fragmentary or not subsequently reported or confirmed. However, whenever claims of DNA-mediated genetic transfers are made, it is necessary to maintain sufficient skepticism until such time as the phenomenon is well substantiated. For example, Gracheva (130) has reported the transformation of E. coli communis to B. breslau by means of heat-killed cells of B. breslau and rabbit serum. Kalina (222) has found that B. breslau is inactivated at higher temperatures than E. coli and that heating to 60°C. for 30 min., the procedure used by Gracheva, would leave viable forms which, if passed through a filter, could give the indicated results.

The transfer of genetic information by means of naked DNA has been most thoroughly studied with two groups of organisms, those related to Diplococcus pneumoniae and Hemophilus spp. Recently, there have been a number of reports on transformations in other organisms, some of which appear to have a considerable amount of support. Balassa (20 to 23) has described a number of experiments with Rhizobium "species" in which there has been a transfer of streptomycin resistance or virulence from one organ-

ism to another. The genetic transfer is destroyed by deoxyribonuclease treatment and is temperature dependent. In a recent abstract (24), Balassa describes the induction of prototrophs from mutants requiring cysteine, isoleucine, and valine. The frequency of transformation in this experiment depends upon the time of addition of DNA, i.e., the competence of the cells is time dependent. "Altered heredity" for *Rhizobium* was described by Krasilnikov (235, 236) and by Peterson (299). Balassa's evidence is convincing, but a little doubt is cast on the phenomenon since Peterson found that he could regenerate filterable forms from old cultures [(299); also see (45)].

A particularly interesting report has been presented by Spizizen for the transformation to nutritional independence of three auxotrophic mutants of *Bacillus subtilis* (333). The reaction appears to depend upon the concentration of DNA and upon the development of competence of the *B. subtilis* cells. Of particular interest is the finding that an enhancement of transforming activity is produced by a ribonuclease-sensitive fraction closely associated with donor DNA.

There have been a number of reports of transformations in Salmonella. Demerec and Lahr have described transformations in Salmonella typhimurium in which the characteristic for streptomycin resistance was transferred into strains carrying auxotrophic markers so that there was little likelihood that donor cells would account for the transformations produced. The phenomenon was sensitive to deoxyribonuclease (73). Fifty per cent of the transformants also showed a requirement for thiamine and some also for nicotinic acid (Demerec and Lahr, personal communication). Transduction tests showed both of these requirements to be associated with or to be linked to the streptomycin-resistance marker. It was suggested that the thiamine requirement might have arisen by damage to the DNA during extraction from donor bacteria (73), an interesting idea which, however, needs thorough confirmation. Kimura (227) reports transformation of Salmonella typhosa by DNA from streptomycin-resistant S-type bacteria, resistant to 10 mg. /ml. of streptomycin. In this case there appears to be an association (linkage) of a capsular characteristic with streptomycin resistance. Linked transformations in Salmonella typhosa from rough to smooth and from streptomycin sensitivity to streptomycin resistance with DNA from the smooth streptomycin-resistant cells, were also described by Kinoshita (228). The reaction is insensitive to ribonuclease but deoxyribonuclease is effective in destroying transformations. Terada (347) describes a case of transformation of white colony Salmonella Elly strain to a yellow colony type by DNA extracted from the yellow Salmonella Kedrowsky strain. Some negative results have been reported for attempts to transform E. coli and Shigella dysenteriae for streptomycin resistance (228).

An interesting review of interspecific transformation in *Hemophilus* spp. has been presented by Schaeffer (323). In addition, interspecific transformations between species related to *D. pneumoniae* are presented by Bracco *et al.* (49) and by Pakula *et al.* (293, 294, 295). In general, interspecific trans-

formations produce lower levels of transformations than specific transformations. Schaeffer suggests that this lower frequency of transformation is caused by a lower efficiency of pairing between the heterologous DNA and the DNA of the chromosomes of the recipient bacteria. This is suggested by two observations: (a) P³²-labelled DNA is taken up equally well from heterologous strains as from homologous strains, and (b) competition for homologous P³²-labelled DNA uptake is the same for specific and interspecific DNA (323). In addition, after (some of) the heterologous DNA molecule has been integrated into a recipient bacterium in transformation, the DNA subsequently extracted from the progeny has the same affinity for the parental recipient bacteria as does homologous DNA, i.e., in their efficiency in transformation, the new molecules behave as homologous DNA rather than as the original heterologous DNA.

An interesting case of a modification in DNA transformation has been reported by Austrian (15). He has shown that a particular mutant type III, when exposed to DNA from type I, will transform to types I and types I–III. But if this same host mutant III is exposed to DNA from type I–III, no I's or I–III's are produced. This suggests that the DNA is altered in such a way that it will not produce a single step transformation to the characteristic I–III.

Catlin has reported that the extracellular DNA derived from meningococcus has transforming activity (58, 59, 60).

Three groups of workers have attempted to determine the dependence of transformation on the size of the DNA molecule. Litt $et\ al.$ (260), using sonic oscillation and an agent to prevent free radical formation, have determined the critical molecular weight required for transformation of streptomycin, erythromycin, or micrococcin resistance in pneumococci. The sonication produces a scission of molecules accompanied by loss in transformating activity. A plot of the relative transforming activity remaining against the reciprocal of the molecular weight is represented by a straight line. An anlysis of these data by the target theory gives a molecular weight value of 1,000,000. The data presented do not distinguish between the possibilities that this is: (a) the minimum size required for attachment to the bacterial site of uptake, or (b) the minimum size for incorporation into the bacterial genome.

Rosenberg et al. (311), have used a mechanical shear device to reduce the size of DNA molecules. In addition, these authors measured the decrease in rate of absorption of P³²-labelled DNA. A large portion of the activity lost after mechanical shear appears to be caused by failure of absorption of the smaller molecules. These authors derived equations which relate the destruction of transforming activity by mechanical shear to the size of the resulting molecules. By combining these equations with kinetic equations for transformation (by the Michaelis-Menton procedure), transformation is described as a function of the molecular sizes of active DNA molecules.

However, evaluation of the conclusions must await presentation of further information on the mechanism of inactivation itself.

Lerman & Tolmach (255) present a model for the action of ultraviolet light on DNA consistent with the data for ultraviolet inactivation of a number of markers in pneumococci. They assume that once a photon has been absorbed, the energy may be transmitted along the DNA molecule to produce damage. The energy of a second absorbed photon cannot be transmitted past this point, so that a second hit must occur between the initially affected structure and the active locus in order to produce a damaged genetic marker. Only a single hit within the genetic region covered by the marker is needed to produce a lethal event. Lerman (254) has combined the ultraviolet inactivation data with other data obtained from titration of acid liberated on hydrolysis of DNA by deoxyribonuclease and from the biological activity associated with the deoxyribonuclease-treated material. He has estimated the size of the critical segment required for transformation of each of several markers to be about 3000 nucleotides (254).

Latarjet et al. (239), have presented a study on the rate of inactivation of the transforming activity by different peroxides. Not all peroxides are effective. The effect of hydrogen peroxide is highly dependent on the purity of the DNA. Of particular interest is the fact that disuccinoyl peroxide, which gives monopersuccinic acid in solution, is 104 times as active as H₂O₃. The amount of monopersuccinic acid required to inactivate transforming activity is comparable to the amount of hydrogen peroxide produced by a dose of x-rays required for the same level of inactivation. Beiser & Ellison (36) have studied the ultraviolet light inactivation of the streptomycin marker in pneumococcal DNA before and after extraction from the cell. They confirm earlier reports that the pneumococcus is not photoreactivable (129). Moreover, they find that the DNA isolated from bacteria irradiated with an appropriate dose of ultraviolet light has no transforming activity, whereas DNA from unirradiated bacteria given the same dose of ultraviolet after extraction loses only 35 per cent of its activity. These results are in contrast to those obtained by Goodgal (128) for Hemophilus where DNA, irradiated either prior to or after lysis, yielded the same relative levels of transforming activity.

Stuy has shown that *Hemophilus* cells, after ultraviolet irradiation and incubation, lyse liberating DNA with lowered transforming activity (342a). The photoreactivation of ultraviolet-inactivated transforming factor has been demonstrated by Goodgal *et al.* (129) and by Rupert *et al.* (315, 316). The reactivating system consisted of irradiated DNA, magnesium, and a cell-free extract of *E. coli*. Photoreactivation can also be demonstrated by means of a yeast enzyme (314). A competition for enzyme is exerted by homologous and heterologous DNA as long as they are pretreated with ultraviolet radiation (314). This suggests that similar substrate(s) for the enzyme is (are) created in different DNA's by the action of ultraviolet light.

Pakula and his co-workers have observed that some preparations of serum albumin are ineffective in promoting DNA-mediated transformation in pneumococci. They observed that an effective albumin preparation was the only one which would produce agglutination of recipient bacteria. DNA-mediated transformation to streptomycin resistance was effected by the addition of antibodies to type R in addition to the inferior albumin preparations. Two strains of *Streptococcus viridans* were isolated which were transformed to streptomycin resistance by means of pneumococcal DNA. These strains would transform in the presence of any of the albumin preparations without addition of antibodies (293, 294, 295).

Kohoutora, in the study of the effect of monovalent cations on transformation of pneumococci, found that sodium or potassium ion was necessary in order to obtain consistent recovery of transformants (231). Ammonium ion could replace these ions to a lesser extent. Lithium, magnesium, and cobalt were inhibitory to the transformation process. In addition, young cultures required a higher concentration of sodium and potassium salts than old cultures. Of interest, too, is the observation that calcium is not required for transformation (231). Perhaps adequate calcium was supplied as contaminating ions, for Fox & Hotchkiss (84) have shown that low levels of calcium are required for transformation to occur.

Goodgal (127) reports that chloramphenicol prevents the development of competence but does not prevent the irreversible uptake of DNA by *Hemophilus* cells which are already competent. The expression of a transforming factor is prevented by chloramphenicol, in agreement with the data reported by Fox & Hotchkiss (84) in pneumococci. Development of expression may continue once again after the chloramphenicol is removed (127).

Austrian et al. (16, 17), have presented a preliminary report of work which promises to place the morphological, capsular transformations in pneumococci into the realm of biochemical genetics. The experiments of this group were an outcome of an investigation into the chemical composition and enzymatic synthesis of the polysaccharide coats of type I and of type III pneumococci [also see (329, 330)]. Following transformation, the presence of substrates and enzymes were detected in bacteria formerly lacking these substances. Correlations were then made between genetic factors and the biochemical steps which they determine. Three main conclusions are presented: (a) Transformation for heterologous capsular type may involve transfer of an entire biosynthetic pathway, i.e., more than one gene locus may be transferred at a time. (b) Some of the intermediates in one biosynthetic pathway may be used in synthesizing both the type I and type III polysaccharides. (c) Genes controlling the elaboration of a single polysaccharide are closely linked, whereas genes controlling the synthesis of two capsular components of doubly-encapsulated strains are more weakly linked.

In another substantial contribution, Hotchkiss & Evans (168, 169, 170) present an analysis of a complex locus involved in sulfonamide resistance in pneumococci. An organism, resistant to a high level of sulfonamide and

obtained in a single step, was demonstrated to contain genetic alterations at three sites separable by recombination. Various combinations of the three sites interact to give different levels of resistance to sulfanilamide.

The relative frequencies of multiple- and single-marker transformations were dependent upon the conditions under which transformation took place and the competence of the recipient bacteria. Since the resistant mutant was isolated as a "one-step" mutation and the three mutational sites are within one molecule or particle, Hotchkiss suggests that all three sites may be contained within a single genetic locus. He adopts the working hypothesis that sulfanilamide resistance arises from altered affinities for drug and metabolite of a p-aminobenzoic acid utilizing enzyme. Considerable help was derived in analyzing the three sulfonamide sites by the varying levels of sensitivity of the mutant organisms to inhibitory analogues of p-aminobenzoic acid. As Hotchkiss points out, the fine structure of this genetic region offers an interesting promise for biochemical study (169, 170).

PHAGE-MEDIATED GENETIC TRANSFER

Special transduction.—There exists but a single locus on the E. coli K12 chromosome at which active λ phage may become localized in the prophage state. The locus has been mapped by a number of methods and found by all tests to be closely linked to some genes involved in galactose utilization [reviewed in (149, 206, 213, 214, 380, 381)]. Lytically-produced λ phage is incompetent in transduction, but rare particles obtained from induced lysogenic bacteria are competent in restricted or specialized transduction of the closely linked galactose genes. Many of the resulting transductional clones are unstable; these bacteria are diploid in the gal region [Morse et al., (276, 277); reviewed Luria (265)]. If these hemizygous bacteria ("heterogenotes") also carry a second, active \(\lambda \) prophage, or if the bacteria are superinfected with such phage, they yield HFT (high frequency transducing) lysates (54, 276, 277, 369). Under these conditions, transducing λ genomes actively reproduce and mature, each burst releasing many transducing particles (369). Such HFT lysates are composed of two types of genetically different particles: (a) nontransducing, "active" particles carrying a complete phage genome, and (b) defective, transducing particles (12, 14, 54, 55, 369). Weigle [cited in (12)], using density-gradient centrifugation (273, 274), has physically separated transducing λ from the nontransducing particles. While normal, active λ and some λ mutants each present a single, sharp band in cesium chloride, HFT λ present four bands of decreasing densities: (a) heavy, active, nontransducing; (b) light, active, nontransducing; (c) heavy, defective, transducing; and (d) light, defective, transducing λ (370). The particles constituting HFT lysates are morphologically uniform under the electron microscope (12, 14). The DNA of λ -phage differs from bacterial DNA neither in base composition [Smith & Siminovitch, cited in (268)] nor in base ratios [Arber, cited in (224)].

The defective, transducing particles require the cooperation (comple-

mentation) of some functional genetic units of a second, active λ -genome in order to transduce efficiently [Arber, (12); Campbell (54)], release intact phage upon induction, or lysogenize (12, 14, 369). That a portion of the λ-chromosome is absent in the defective, transducing phage is inferred by its: (a) failure to complement certain specific genes; (b) failure of certain specific genes of the transducing defective to appear among the recombinants with active λ or closely related phages; and (c) failure to otherwise determine the presumed products of genes in the defective region. For example, a heterogenote, obtained after infection with a wild type λ -lysate, upon induction and lysis releases no serum-blocking antigen nor any morphologically discernible phagelike structures. Upon superinfection with a λ hostrange (h) mutant, neither the h^+ phenotype nor the h^+ genotype is present in either the active or the defective phage released in the mixed bursts. The conclusion is made that the h gene, along with others, is absent in the defective particles, being replaced by a gene region (dg) containing galactose genes derived from the host. The genotypes of the predominant segregants (54, 56, 277) suggest that the phage genome is physically attached to the gal genes. Further, the defective region maps centrally in the phage genome, with phage markers located at either end (12, 55, 56).

Markers available for a large number of regions of the λ chromosome (218, 219) have recently been augmented by discovery of host-dependent mutants (55, 56) and mutations critical at various single stages of vegetative reproduction and phase maturation (13, 207, 208, 374). An adequately marked phage genome allowed Campbell & Balbinder (55, 56) to show the existence of four different species of transducing particles. The dg region varies in extent at one of its ends in the different clones of transducing particles. Both Campbell (55, 56) and Arber (12) find that the other end terminates uniformly, although adequate markers are not yet available to decide this point with certainty. In contrast, each clone, derived by growth following transduction with a "normal" λ lysate, consists of but a single genetic species of defective particles.

The dg region does not appear to recombine with the active λ genome (12), although special techniques might be required to demonstrate such recombination (presumably the production of nontransducing mutants) if it occurred. The dg region may, however, recombine with bacterial gal genes, giving rise to stably integrated gal recombinations (12, 14, 54, 276, 277). From the few markers utilized in the genetic tests (op cit.) it may be assumed that the dg region contained in several independent HFT lysates encompasses at least two functional units of two gal genes [cf. 220, 221, 237)]. Additional markers are now available for the gal region (241); these, together with biochemical tests, should allow insight into the fine structure of the gal region of the host chromosome as well as the dg region of the various HFT derivatives.

Normal λ prophage appears to hinder integration of input gal markers; λ -dg prophage exerts an even stronger, if not complete, "steric interfer-

ence" (12). Perhaps input λ -dg, unlike active λ , has a choice of two short chromosomal regions with which to synapse, i.e., the λ locus or the gal loci. Consistent with this idea is the observation that bacteria doubly lysogenic for λ -dg (immunity λ and immunity 434) frequently lose both prophages simultaneously (56). Perhaps the localization of the genetically different particles could be elucidated through the use of heavily P^{32} -labelled phages, frozen at an opportune time following infection, and the bacteria examined for mutation at the appropriate region (λ , 434, or gal loci), or for marker transfer in conjugation experiments.

General transduction.—Some bacteriophage particles, when obtained either from sensitive bacteria through lytic passage or when obtained from induced lysogenics, contain various limited portions of the genome of the lysed bacteria. The Salmonella phage, P22, is the classic example (391), although many other phages competent to carry out this type of transduction are now known for enteric and other bacteria. The loci transduced collectively encompass a large portion, if not all, of the bacterial genome [reviewed in (125, 149, 387, 390)]. This has enabled extensive studies of many gene regions in Salmonella [see (72)]. Several markers for antibiotic resistance, previously thought to be nontransducible in Salmonella strain LT-2 (149), have been found to be transduced in studies utilizing improved technical measures which allow adequate phenotypic expression [e.g. (73, 367)].

A few special markers have been found to be nontransducible. Ozeki has considered the case of a stable mutant, ade-3, described by Yura (385) and attributed it to a chromosomal aberration (292). Another adenineless mutant, similar in behavior, has been described by Furness & Rowley (117). Hartman et al. (151) have noted an instance of a nontransducible histidine mutant (his-57) which is a probable inversion; the mutant is stable to back mutation, is nontransducible, may act as a donor for certain genes, and is deficient in a number of enzyme activities. Bailey (18) described a peculiar instance of failure to obtain transduction with a particular marker, although both the reciprocal test and other donors yielded recombinants; in this case, the particular marker also spontaneously reverted. Furness & Rowley (117) failed to transduce the property of virulence into a prototrophic Salmonella strain. Perhaps the failure in this instance, as well as those described by Ozeki and by Bailey, results from double mutation. Polygenic systems involved in multistep penicillin and chloramphenicol resistance have been transduced but require special techniques for their demonstration (26, 73).

The frequency of transductional clones recovered per given number of phage particles for a single marker may vary with experimental conditions, for example, the time during the latent period when the phage is harvested (387) or the state of the recipient bacteria and plating conditions [discussed in (149)]. Similarly, the frequency of transduction varies between markers located in different regions of the chromosome. This, again, may be attributed to factors influencing "gene pick-up" during single-step phage growth in the donor host (387), to the positions of the markers relative to

the ends of transducing fragments of uniform sizes and compositions (292), physiological conditions of the recipients (148, 149, 151, 379), or the precise nature of the mutational changes being relieved by transduction (148, 151). The frequencies may be differentially modified for different markers in the same gene region by treatment of the recipient bacteria with cations or with chelating agents (229, 230, 312).

Garen & Zinder (119) showed that light ultraviolet irradiation of transducing phage prior to infection increased the number of complete transductions recovered; this has been confirmed for a number of different markers [Ozeki (in (74); (150)]. Further irradiation of phage grown on wild-type bacteria results in a slow, exponential decrease in the number of recombinants obtained. The rate of the decrease is uniform as long as the strains bearing single-site ("point") mutations are utilized for the assay, but it progressively increases as multisite mutants, requiring larger and larger segments of wild-type genome in order to produce a detectable recombinant, are utilized (151).

It is claimed that frequencies of transduction for different markers bear a relation to the chromosomal location of the prophage of the transducing phage (300, 301, 310). There are now available a number of experimental methods capable of testing this interesting theory [which is not unlike a possibility earlier suggested by Bertani (42)]. The data presented so far by Plough and his co-workers, however, do not contribute an answer to the question. Only one of fifteen temperate phages studied which lysogenize E. coli K12, did not show a chromosomal location in conjugation experiments [Jacob (206)]. This phage is uniquely able to participate in general transduction (204).

Winkler & Kaplan have presented data indicating that various transducing phages fall into two general classes with regard to transduction frequencies under standard conditions (377). Demerec *et al.* (73) find that phage P1 probably transduces a larger chromosomal segment than does P22. It may be that phages effective in general transduction carry either single units (DNA molecules?) or two such adjacent units of host material.

Lennox (252), using P1 phage, and Jacob (204), utilizing a closely related phage, first demonstrated general transduction in $E.\ coli$. Following the discovery of defective particles involved in λ -gal transduction (see special transduction), close examination was made for the occurrence of a related phenomenon in a system giving general transduction. Adams & Luria (1) carried out an ingeniously devised and careful analysis of transduction by P1 phage. The phage was probably chosen for examination since: (a) unlike many temperate phages [e.g., Salmonella A phages: (86, 258, 267, 389); λ : (259, 267); other phages: (267, 344, 345)], P1 phage is rapidly reduced to prophage, failing to segregate off sensitive bacteria in the initial divisions following infection (1, 126); (b) Lennox (252) had noted a negative correlation between lysogeny for P1 and transduced characters in transductional clones as well as the presence in certain transductional clones of defective lysogenics (1); (c) Lennox (252) had also noted the presence of unstable

transductional clones; and (d) adsorption and infection by P1 phage requires calcium, whereas P22 phage is rapidly adsorbing under a variety of conditions, allowing superinfection on the plate. With E. coli as donors, Adams & Luria (1) found that the transductional clones of E. coli and Shigella recipients were usually stably P1-sensitive and nonlysogenic where conditions of single infection were assured. E. coli lac+ were also stable and composed of P1-sensitive bacteria. In contrast, unstable lac+ P1-immune clones were detected in Shigella recipients; the lac- segregants from the unstable clones simultaneously became P1-sensitive. The joint loss of these two properties was interpreted as indicating a physical attachment of phage and bacterial genetic material (1). Lysates from the unstable, immune clones have yielded transducing phage which is 104 times more efficient, per plaqueforming unit, than the initial infecting phage particles (originally 10-6 to 10⁻⁸ transductions/phage particle) (267). Luria et al. interpret the detectable persistence of the defective transducing element as attributable to an impaired ability of the genetic fragment to persist as prophage (267). This is presumably coupled with genetic dissimilarities between E. coli and Shigella in the lac region, also impairing complete integration of the transduced bacterial markers. Interesting differences in genomes at the lac region were also noted in conjugation studies by Luria & Burrous (266).

Starlinger (335) has presented evidence consistent with the view that general transduction in Salmonella with P22 phage is also mediated by defective particles [also see (63)]. However, because of the technical problems inherent in this system, Starlinger's interesting findings require further confirmation by other methods. Prior experiments by Zinder (387, 390a), while cleverly initiated, had failed to provide an answer on this very point. The presence of multiple effects of the phage genome during the period of prophage stabilization, for example, paraimmunity (267, 389), antigen conversion (see later discussion), and the blocking of synthetic reactions [e.g. (114)], give promise of indirect determination of the defective state. As Luria et al. (267) have pointed out, however, thorough verification must lie in genetic analysis of a system where rapid integration is impaired.

Witkin & Thomas (379) noted that prototrophs comprised but from 0.01 to 82 per cent of the bacteria in transductional clones isolated in non-selective media. By plating on media with varying degrees of limiting enrichment, Witkin and her co-workers (378, 379) found that prototrophs were expressed with but little nutritional aid; the experiments failed, however, to provide critical evidence on the timing of the integration events. Demerec et al. (73), following transduction with P22 phage, have discerned clones which are mixed with regard to two linked markers (a fermentation and a nutritional marker), i.e., two different recombinant types were obtained from a single clone. Upon restreaking, such clones have so far been found to be composed of stable segregants. Hartman et al. (151) also noted a similar instance of mixed clones for a different bacterial gene region. The input fragment thus appears to be capable, in a limited percentage of cases, of under-

going several rounds of mating but, as yet, no stable "heterogenotes" have been detected in the *Salmonella* system. Possibly the unstable transductional clones described by Spicer & Datta (330a) might prove to be of this nature; if so, where two functional alternatives exist, the input genes must be epistatic to those of the recipient chromosome.

Transduction frequency is proportional to phage input over a range of multiplicities from 0.01 to 1; the efficiency drops slowly at higher multiplicities and decreases rapidly at multiplicities greater than 50, presumably caused by lysis from without. Phage killed with ultraviolet light to 10^{-4} to 10^{-5} survival still show a yield of recombinants on antiserum plates proportional to phage input and is insensitive to assistance by active, nontransducing phage (151).

Bhaskaran (44) has claimed general transduction in Vibrio, Loutit (261) for a Pseudomonas aeruginosa strain, and Ritz & Baldwin (309) for Staphylococcus pyogenes.

No attempts have been reported concerning the ability, or inability, of naturally "lytic phages" to transduce or to convert. Such activities by defective transducing phages could theoretically be observed in the survivors of single infection in the presence of antiserum or where infection is abortive [e.g., in the systems described by Lederberg (250)].

Abortive transduction.—In abortive transduction the phage-transported chromosomal fragment is injected into the recipient bacterium, functions, but neither replicates nor is integrated into the replicating bacterial genome with high frequency. First noted for transduction of motility-conferring factors (244, 339, 342), the analogous phenomenon has been described for auxotrophic markers by Ozeki (291) and others. Ozeki's results (292) indicate that the genetic fragments participating both in complete and in abortive transduction are probably uniform for each gene region, i.e., are all of the same length and composition. Where linkage is found by complete transduction, it is also found in abortive transduction. Furthermore, pairs of genes known to be linked by recombinational analysis in complete transduction are always transferred together in abortive transduction [(291); also see data in (244)]. A more sensitive test of this concept is desired.

Eisenstark and his co-workers (78, 312) find that both complete and abortive transductions are equally altered by ultraviolet irradiation of transducing phage or by various treatments of recipient bacteria. In contrast, Ozeki (291) and Hartman et al. (150) find abortive transduction to be more sensitive to ultraviolet inactivation than is complete transduction. In the inactivation of his+ clones, there is a single-event, exponential decline in abortive transduction frequency with an increasing ultraviolet dose; the rate of inactivation parallels the straight-line portion of the inactivation curve for plaque-forming units. For other genes, the rate may be somewhat lower. The inactivation rate for the formation of his+ clones is the same for his recipients requiring but one functional genetic unit and for those requiring more (up to 10); it is about 40 times the rate of inactivation found in

the logarithmic portion of the curve for complete transduction of single-site markers. The "multiple hit" curves for inactivation of complete transduction extrapolate to values which vary slightly about nine. The same phage preparation, unirradiated, yields eight abortive transductions for every one complete transduction. Hartman et al. (150) have suggested that absorption of a single photon alters a fragment, otherwise destined for abortive transduction, allowing it to participate in integration. Specific selection by linked transduction for fragments, each of which has several such "hits," reveal no induced mutations among the complete transductional clones.

From occasional lysates one may obtain ratios of abortive to complete transduction of 30:1, compared to the normal ratio of 8 to 10:1. This variation holds equally for different markers, whether linked or unlinked, even when the frequencies of transduction of the markers differ by as much as two decades (Hartman & Hartman, unpublished).

Abortive transduction offers a rapid test for functional allelism, applicable to almost all gene loci of the bacteria [e.g., Demerec et al. (72, 73)].

Phage conversion.—Upon penetration of a phage genome into a host, a host-virus complex is formed. Some of its new attributes are associated clearly with the genome of the "active" phage and some of the new traits are clearly derived from the preceding host cells. The dividing line becomes less clear, however, when we review the features of special transduction where the defective phage genome carries bacterial genes in place of some of its own genes. In addition, other alterations are imposed by certain phages upon the cells they infect. It was at first thought that these changes were characteristic of the prophage-bacterium complex and the phenomena were collectively called "lysogenic conversion." It has now been discovered that the altered properties do not depend upon reduction of the infecting phage genome to prophage, hence the term "phage conversion." Discussion of phage conversion is provided in recent reviews (2, 43, 149, 265, 269).

Antigen conversion.—The phage-bacterium complex in certain cases forms a specific somatic antigen not present in the uninfected bacterium. Sometimes somatic antigen(s) previously produced by the bacterium is no longer elicited by the complex; if the phage genome is lost from the cell, however, the bacterium again produces the antigen initially present before infection. The phage genome appears to induce the production of antigen when in each of its forms (infecting phage, vegetative phage, prophage, etc.).

Salmonella strains producing the antigens 3, 10 may be converted to produce the antigens 3, 15 by lysogenization with ϵ^{15} phages arising from 3, 15-producing or 3, 15, 34 bacteria (115, 145, 146, 175, 176, 184 to 187, 283, 287, 318, 319, 360, 361). Similarly, bacteria producing somatic antigens 1, 3, 19 can be converted to produce 1, 3, 15, 19 [(175, 188, 189); also see (3, 4, 173, 174, 190, 360 to 363)]. Probably 3, 10, 26 bacteria were also converted to 3, 15 production by these same phage (184), although phage was not directly implicated in early reports (52, 364, 383). The ϵ^{15} phages represent a distinct group of phages, similar in serological characteristics, plaque

morphology, host range, etc. (356). Antiphage and anti-3, 15 (containing antiphage) antibodies allow the selection of nonlysogenic derivatives from from 3, 15 populations; these strains now again produce antigens 3, 10 (188, 189, 361). Of six *Escherichia freundii* strains with the antigenic constitution 3, 10, Uetake was able to convert one to antigen 3, 15 production with ϵ^{15} phage (356). An *E. freundii* strain was also found with the antigenic structure 3, 15; it failed to produce detectable ϵ^{15} phage and no 3, 10-producing derivative could be obtained by antiserum selection. Certain *E. freundii* strains produce a bacteriocin (colicin A) but data on the antigenic constitutions of these were not given (108).

The ϵ^{15} phages conserve their ability to convert after passage through 3, 10-producing bacteria, indicating that the induction of antigen 15 production is a quite stable property of the phage [e.g. (319, 361)]. The new antigen is produced soon after infection and before cell division ensues, initially resulting in a mixture of both antigens 10 and 15 on the cell surface. Antigen 15 is elicited equally well in this early period by infection with the temperate ϵ^{15} phage or with virulent mutants derived therefrom (356, 357, 359). The antigen is detected serologically and by the use of ϵ^{34} phage, which utilizes antigen 15 as a receptor site for adsorption. During infection with the temperate phage, where reduction to stable prophage is delayed for several divisions, nonlysogenic, sensitive bacteria are segregated which revert to antigen 10 production [Uetake, Luria & Burrous (359)].

A few naturally-occurring strains are known which stably produce both antigen 10 and antigen 15 simultaneously (280, 361). Three types of bacteria have been obtained from a strain carrying e15 phage and producing only the 3, 15 antigens: (a) bacteria which are sensitive to ultraviolet light and retain antigen 15 but which, upon lysis, release no phage; (b) those which are no longer sensitive to ultraviolet light and produce no phage but retain antigen 15; and (c) those which retain capacity for ϵ^{15} production and antigen 15 production but which are noninducible; phage from this defective strain, when infecting sensitive bacteria, give rise to inducible lysogenics [Uetake cited in (265); (358)]. The existence of the third strain indicates that (in addition to the prophage genome) the bacterial host plays a role in inducibility [compare (171)]. The first two categories possibly represent varying degrees of defective lysogeny; in these cases it may prove difficult to differentiate such a state from an ordinary bacterial gene, guiding the synthesis of a "normal" bacterial constituent. Both antigens 10 and 15 are constituents of the cell wall but are also found to be present on "protoplasts" after removal of at least the majority of the cell wall material (359).

In contrast with the capacity of almost all active particles to convert, only a small proportion of ϵ^{15} particles in a lysate have been reported capable of general transduction (176, 188, 319, 325, 360). Transductional clones are not always lysogenic nor do they always show antigen conversion (176), although the very early stages of the process have not as yet been thoroughly studied.

A second group of phages, serologically unrelated with e15 phages, adsorb to antigen 15 and elicit the production of antigen 34. Thus ,3, 15 bacteria are converted to 3, 15, 34 (145, 146, 177, 281, 282, 357). Since different e^{34} phages form two different plaque types, the conversion is attributed to only a portion of the phage genome (357). Treatment of the culture containing ϵ^{34} prophage with anti-34 antiserum allows the isolation of non-34-producing bacteria which are no longer lysogenic for ε³⁴ prophage (146). ε³⁴ Phage is also

capable of low-frequency general transduction (177).

A third group of serologically closely related phages induce the formation of antigen 1. These phages are variously called iota, or A1 and A2, or heatstable A phages (175, 178, 179, 180, 347, 357). The phages, like P22 transducing phage (391), adsorb to a form of antigen 12. The conversions are thus from antigens 9, 12 to 1, 9, 12; from 4, 5, 12 to 1, 4, 5, 12; or from 4, 12 to 1, 4, 12; etc. Particles from all single plaques tested convert (340). When the prophage is lost from lysogenic bacteria, production of antigen 1 ceases. The new antigen is detectable well before the end of the initial latent period with either temperate or virulent phage (340, 341, 388, 390a), and is not detectably present in either exposed or masked form in the nonlysogenic bacteria prior to infection (388). Not all naturally-occurring Salmonella strains carrying antigen 1 spontaneously release active converting phages (357). Some of these strains yield low numbers of phage particles upon induction (390a); in other strains the presence of portions of phage genome is detectable by phage recombination tests [Zinder (390a); compare (65, 389)]. Bacteria carrying antigen 1 can be differentiated from nonproducing-1 cells by colonial morphology on antiserum plates (183).

Antigen 1 is present in the cell wall and can be extracted; the haptenic polysaccharide of the converted cells is precipitated with anti-one antiserum (341). The antigen can also be detected in protoplasts [Zinder cited in (359)]. Hydrolysates of the polysaccharide from the lysogenized bacteria show differences from those obtained from the sensitive bacteria (341). The polysaccharide constituent of antigen 1 appears to be composed of glucose residues, linked in α 1-4, with a terminal residue of a deoxymethyl-

pentose (336).

One of the A1 phages, P22, has been extensively used in transduction experiments (72, 391). Other antigen 1 converting phages also exhibit general transduction (181, 347). In some cases, this transduction is not accompanied by detectable antigen conversion (181). Possibly, in these latter cases, abortive infection occurs, similar to that found by Zinder with P22 phage after growth on Salmonella gallinarum [Zinder cited in (119, 264)].

The genetic structure of one of the antigen 1 converting phages, P22, has been analyzed more extensively than that of any other converting phage (258, 389). The genetic unit which confers the capacity for antigen 1 production constitutes just a portion of the phage genome, probably a single locus (345, 346, 348, 388, 389). It is genetically inseparable from a locus controlling the serological specificity of the phage (390a). Sometimes antigen 1 production exhibits "form variation" (incomplete penetrance), being expressed by only a fraction of the bacteria in a population (341). The amount of antigen produced (expressivity) is dependent in part upon the phage genotype at the presumed "antigen-1" locus (345, 246, 348).

Antigen 20 may be conferred upon some Salmonella strains by lysogenization with specific phages (11, 33). Bacteria with the antigenic structure 8 or 6, 8, upon lysogenization become 8, 20 or 6, 8, 20, respectively. The phage also confers immunity and the bacteria produce active phage, often with altered host range properties [compare Zinder (389)]. In the case of 8, 20 bacteria, the phage now also fails to adsorb.

Lysogenization with A3 and A4 phages reduce greatly the adsorbing capacity for A1 phages (48), including P22 phage (340). An analysis of this

change in surface properties has not yet been made.

Antigen conversion by phage has also been reported for Shigella (271). The possibility exists that the antigen found in Shigella-E. coli hybrids by Luria & Burrous (266) is attributable to the presence of a defective prophage near or at the lactose locus and is either lost from the Shigella recipient during recombination, allowing expression of another antigen, or is inserted from the E. coli donor. Iseki & Nagaseki (182) reported the formation of a unique antigen in E. coli hybrids. Lysogenization in Pseudomonas has also been reported as accompanied by antigen conversion as well as by changes in plant pathogenicity and carbohydrate utilization (289, 352 to 355). Tsujita & Matsui (354) note that, following lysogenization, only very slow growth is obtained on mannitol or lactose. Nonlysogenic derivatives, obtained after ultraviolet irradiation, are again able to grow well on these two substrates. In addition, derivatives are obtained which grow well either on lactose or mannitol; these strains have all lost the ability to release phage spontaneously. Further, a galactose cryptic was converted by lysogenization to galactose positivity.

The above results may be compared with those of Lederberg (240) who showed that mutation to λ -resistance resulted in a simultaneous loss of ability to utilize maltose. One temperature-sensitive allele was noted. Her abstract does not state whether the maltose negatives at this locus are cryptics of if maltose uptake is adaptive. She notes, however, that the presence of maltose or amylose does not influence λ adsorption. It is possible that specific permeases (66) may be altered or differentially shielded from effective positions by other, equally specific, alterations in the structure of the bacterial cell. While permeases are, at least in part, associated with protoplasts (50, 307, 326), in some cases the cell wall also appears equipped to aid in uptake

(51).

The serological and phage-adsorbing properties of the bacterial surface have received concerted attention in particular instances because of epidemiological significance. Only recently is evidence accumulating on the chemical composition of specific portions of the surface mosaic with regard to particular phage receptors [e.g. (124, 217, 320, 368)], specific antigens de-

tected by serological methods (76, 217, 285, 371, 372, 373), or colicinogenic properties of the bacterial strain (7, 34, 35, 120 to 123). Electronmicroscopy of the cell envelop has reached an advanced stage (225). The polysaccharide-containing antigens of enteric organisms are localized mainly in the cell walls, but are also found associated with protoplast interiors and membranes (57, 304, 359). In some cases, it appears that the polysaccharide haptene may be synthesized in the absence of protein synthesis (232, 375, 376).

Enzymological knowledge of polsaccharide synthesis is developing rapidly (334), which should help considerably in the elucidation of the mechanism

and biochemistry of antigen conversion.

Toxigenicity of Corynebacterium.—Certain strains of Corynebacterium diphtheriae have the ability to release toxin under suitable conditions of culture. Particular phages obtained from toxigenic strains, upon lysogenization of nontoxigenic bacteria convert these bacteria to the capacity for toxin production [reviewed in (149); also see (29, 284, 321)]. Detectable phage production is sometimes absent from freshly-isolated (135) or converted (47) toxigenic strains. These strains are generally resistant to temperate converting phage but are lysed by a virulent mutant. Such strains are probably defective lysogenic immunes since known defective lysogenics are high toxin producers. Furthermore, proflavin, which inhibits phage maturation, may increase toxin yield (30).

Contrary to earlier reports [e.g. (153)], iron deficiency, which is essential for high toxin production (384), increases the spontaneous induction frequency of lysogenic bacteria by one hundred times. Since the burst size is also reduced (ten times with the particular strain examined), the instability is partially masked [Barksdale (30)]. The kinetics of toxin release during a single-step growth curve with a virulent mutant of toxin-elicting phage, demonstrate that lysogenization is not a prerequisite for toxin production, just as phage maturation per se is not required (30). Barksdale (30) suggests that the toxin may be derived from a part of the bacterium, split off by a specific enzyme elicited by the phage and responsible for phage liberation or cell lysis or both. The toxins released from different serological types appear qualitatively alike and toxigenicity does not seem to be related to any morphological or biochemical property of the bacteria (278), except for the presence in many instances of detectable prophage. Studies of the composition of the cell walls of Corynebacterium have been reported (69, 70, 71, 162) but not correlated as yet with phage sensitivity or sensitivity to toxin production during phage growth. Toxin production from cell walls treated with phage "lysin" also remains to be experimentally demonstrated. Lysogenic toxigenic bacteria in vivo are selected against, allowing the appearance of nonlysogenic, atoxigenic derivatives [(9); also see (278)]. This effect can be duplicated in vitro through treatment with antiphage antiserum (9).

Groman & Memmer (135) showed that converting phages of independently-derived cultures form a rather uniform group, with regard to host ranges, cross-immunity, and, especially, their serological properties. As prev-

iously noted in other studies [e.g. (298)], some important exceptions were found for host range and immunity. The ability to confer toxigenicity can be separated, by recombination, from the genetic factors responsible for the plating characteristics of the phage (133, 134). Furthermore, phage released from one strain was unable to lysogenize with detectable frequency the nontoxigenic host tested (135). However, toxin was elicited from a mixture of the phage and the sensitive strain, similar to Barksdale's findings reported above.

Phage from certain nontoxigenic strains may convert certain other nontoxigenic strains to toxin production (132, 297). Perhaps toxigencity is not elicited by a unit factor of the phage but by two factors (recombination) or two functional units (complementation). The results reported by Hewitt (158) suggest that the converting phage may have segregated from doubly lysogenic bacteria. Perhaps the second prophage excludes the vegetative growth and function of the converting phage or lyses the cell by an enzymatically different mechanism. That genetic factors of the host bacterium also contribute directly to toxin production is also not excluded. These various

possibilities are amenable to resolution by genetic tests.

Other possibilities.—The properties of Bacillus megaterium strains have been reported to be altered following lysogenization [reviewed in (149)]. McCloy (272) reports that an alteration in colony morphology with one strain was caused by the appearance, in aged cultures, of virulent phage mutants which attacked the otherwise immune lysogenics. Thus, it appears that this phenomenon results from phage mutation rather than from phage conversion. Differences in growth behavior have also been noted between supposedly bacteriocinogenic and nonbacteriocinogenic strains of B. megaterium (201). Interpretation here is tenuous since one may obtain from certain B. megaterium strains premature lysis, resulting in abortive infection or abortive lysis following induction (172). The release of immature phage, phage fragments, or phage lysin from the vast majority of bacteria may mimic the release of bacteriocin (if not be equivalent to it).

Mudd (279) has lucidly pointed out how limited is our knowledge concerning the biology of Staphylococcus. Pathogenic staphylococci are classed into three main groups both by serological tests [e.g. (68, 288)] and by lysis with bacteriophages [e.g. (8, 27, 202)]. There is a also a strong correlation between phage group and antigenic structure of coagulase formed (28). Although limited work has been performed with staphylococcal phages [see references in (2, 257, 302, 313)] and bacteriocins (87, 89, 137, 138), the relationships of these agents to the many toxic agents produced by staphylococci have recevied no attention. Duthie (75) found that coagulase is present in two serologically distinct, yet closely related, forms; one bound and the other free. Bergdoll (39) reported that Staphylococcus enterotoxin is a water-soluble protein, rich in lysine and resistant to trypsin; these are characteristics of cell surface constituents of bacteria.

Naturally occurring pathogenic "Staphylococcus pyogenes" is separated from nonpathogenic species by several seemingly independent, yet highly associated, characters (81). Baldwin (25) noted that Staphylococcus strains produced variants, with a relatively high spontaneous frequency, involving concomitant changes in four traits: production of coagulase, α-hemolysin. mannitol fermentation, and biotin requirement. Selection for the variants could be achieved by several independent means; presumably phage resistance could also be utilized for selective isolation of the same variants. The frequency of appearance of the associated changes was increased following ultraviolet irradiation. The four traits were never observed to change independently. Pigment production was also altered in many of the variants but could also change independently of them. No reversion of any of the four "linked" properties was observed in extensive trails although independent reversion of pigment production was noted (25, 131), Ritz & Baldwin (309) recently have reported phage-mediated transduction of capacity for penicillinase production in Staphylococcus. This observation would seem to release the staphylococci for genetic analysis at a second level, the level of host-phage interaction having been available for analysis for some time.

Raettig (302) lists a potentially useful compilation of changes associated with bacteriophage infection. A large number may be proposed as attributable to phage conversions of various types. However, in most cases, reanalysis is required in order to differentiate between mutation-selection phenomena and alterations induced by phage-carried genetic material. Conjugation and transduction studies between E. coli and Shigella indicate that at least some lactose-negative Shigella are potentially lactose-inducible but defective at the galactoside-permease locus [data in (253)]. A similar defect is found in mutants of E. coli [summarized in (66, 149, 205, 308)]. This is not surprising in view of the crypticity of lactose-negative Shigella sonnei strains which contain a protein immunologically related to β -galactosidase (67), have their naturally occurring lactose-positive counterparts (82), mutate to lcatose positives, and are reported to ferment lactose under special conditions in phage infection (89, 111, 112, 113). It is interesting that in the latter case, lactose fermenting colonies appear in plaques of T3 phage; upon retesting, the bacteria in the colonies appear to be T3-sensitive. Perhaps these clones were transiently infected with semitemperate mutants of phage T3, similar to those found by Fraser (85). Fredericq (89) further observed that a paracolon simultaneously acquired a sensitivity to bacteriocin C, failed to produce this bacteriocin, and became lactose-negative.

CONJUGATION

The discovery of "sexual recombination" in *Escherichia coli* was followed by the accumulation of a number of important yet, at the time, enigmatic observations on the recombination process [reviewed in Lederberg & Tatum (249)]. Resolution of many of the puzzling observations came about with the findings that: (a) the genetic transfer is polarized, going from donor bacteria (designed F⁺ or, for high frequency of transfer, Hfr) to recipient (F⁻) bacteria (154, 155, 162, 246, 382), and (b) only a portion of the haploid comple-

ment is generally transferred [reviewed in (149, 156, 205, 215, 303, 382)]. Recently published results demonstrate that in the average transfer less than a haploid equivalent of P^{32} -labelled DNA is detectably transferred; the transfer of DNA- P^{32} occurs only from the Hfr to the F^- bacteria [Garen and Skaar, (118)]. In pedigrees, only the F^- exconjugant yields recombinants; only one family of recombinant clones arises from a single mating event (10). In contrast to these results, Bernstein (41) states that the F^- partner may act as donor under some conditions in $F^-(\times)F^+$ crosses.

Two of the most striking features of the unidirectional transfer are the slow penetration and the precise orientation of the genetic fragments, allowing detection of sequential transfer of linearly arranged genetic determiners (214). It was at first believed that the genetic fragment donated by a particular Hfr strain began at a fixed point (O or origin) and predominantly terminated at a second fixed point on the chromosome (point of rupture). Recent data of Jacob and Wollman indicate that O, the starting point of the transfer, is the only fixed point and that the size distribution of the piece transferred varies with experimental conditions caused by random breaks or by "spontaneous" separation of the mating pairs during transfer (215, 381). Thus, in rare conjugants, the entire haploid genome may be transferred (206, 215), P³² decay occurring in the DNA of labelled Hfr donors prior to mating may cause still further breaks in the DNA chain and thus further selectively decrease the transfer of markers distally located on the donor segment during the oriented transfer (114a, 214). Under suitable conditions, the transfer of an inducible prophage into a nonimmune recipient bacterium results in zygotic induction and consequent additional elimination, after transfer, of markers closely linked with or distally located to it (213, 215 380, 382).

Once transferred, any single character has about the same probability of integration into the replicating recipient chromosome where the bacteria are relatively isogenic (215, 381). The above methods thus allow genetic mapping by alternative means: (a) time of transfer from a given Hfr donor; (b) frequency of transfer under standardized conditions or after allowing P³² decay to take place in the Hfr bacteria before mating; (c) linkage with a selected marker located distally to that with which linkage is being examined; (d) elimination through zygotic induction with any of a number of inducible prophages located throughout a large portion of the chromosome length; and (e) joint elimination of markers through P³² decay of the donated fragment after transfer into the recipient bacterium. Since the transferred chromosome enters at a constant rate for at least one-half of its length, genetic and physical measurement of linkage can be related to one another (381).

A number of Hfr strains have been isolated from F⁺ cultures and found to initiate transfer at different places in the genome. Results obtained with several independently derived Hfr strains, transferring with high frequency different, overlapping portions of the K12 genome, have allowed Jacob and Wollman to map many widely used genetic markers. The reconstruction of the entire chromosome, based on genetic inference from the tester Hfr strains,

is circular (214). The gene sequence conforms to that deduced from earlier studies (149). The same entire sequence may be demonstrated with a single Hfr donor when special care is taken to allow complete transfer and selection is made for distally located markers (215). The data with different Hfr strains indicate that the Hfr mutation determines where the chromosome terminates and, at the same time, allows the opposite end of the linkage structure to penetrate first into the recepient bacterium. The further question arises: what are the gross structures of the F⁺ and the F⁻ chromosomes?

While the mode of transfer of the genetic material appears to be, in large measure, responsible for the "linkages" analyzed to date, further assortment of the markers takes place following their deposition into the recipient bacterium. Earlier studies on the composition of recombinant clones had indicated that, where the two partners were of different origin [for example, E. coli $B(\times)E$. coli K12], integration into the replicating recipient chromosome is often delayed and the input fragment may undergo several rounds of mating with the chromosome of the recipient bacterium (53, 327). These suppositions have been strikingly confirmed by recent studies of precise pedigree analysis [reviewed in (10, 303)]. Anderson (10) finds that both partners may increase in size and divide during conjugation and after separation. Postzygotic division is irregular, producing from each F ex-conjugant an elongate cell from which a variety of bacterial genotypes and phenotypes segregate: (a) F parental types, (b) morphologically altered types but with the F genotype for the markers examined, (c) recombinant types, and (d) many nonviable bacteria (10). The elongate bacteria and their nonviable progeny are reminiscent of E. coli following ultraviolet treatment (152). Segregation from the elongate bacterium of pure recombinant clones occurs only after three to greater than nine divisions. The ex-conjugant may eventually segregate six (and probably, in some cases, more) recombinant types. The clones of recombinants do not contain (viable) complementary recombinant types. The pattern of origin of recombinant types, as well as their genotypes, have led Anderson (10) to propose: (a) that the input fragment persists in a single line of descendants, and (b) that portions of it may be conserved during recombinational events to undergo multiple matings with the recipient chromosome. The first proposal is supported by data of Fuerst et al. (114a, 214), which shows that the Hfr material remains susceptible to P³² decay for some time after transfer to recipient bacteria. When the two conjugants are of dissimilar origin, fragmentation of the input piece appears to be even more pronounced, for example, in E. coli B(X)K12 crosses (53). Shigella (266) and Salmonella [(31, 32); Demerec et al., personal communication] mate effectively with E. coli Hfr strains but the ultimate number of recombinants recovered is several decades lower than in K12(X)K12 crosses. These results are analogous to comparisons of specific and interspecific transformations (see Transformation).

In particular crosses, at least a portion of the input fragment may persist and multiply in synchrony with the bacterial chromosome (242, 266). Luria & Burrous (266) have found that the stability of this hemizygous ("heterogenotic") state arises from an alteration ("Het" mutation) at a specific site on the donor chromosome at, or very near to, the region which has the special facility of persisting in the heterozygous condition.

Only certain strains of Salmonella are able to form recombinants in matings with F+ of Hfr E. coli strains [(31, 32); Demerec & Miyake, personal communication]. Demerec & Miyake have found that this ability, within Salmonella strain LT-7, resides in a mappable chromosomal factor which elicits other discernible effects on the bacterium. The ability to participate in recombination through conjugation is thus an active, genetically-controlled state for the F- partner. So far it has not been determined if the chromosomal factor is necessary for cell fusion or acts at a later stage in the process. Lederberg & Lederberg (247) have mentioned the existence of similarly refractive strains of E. coli, including derivatives of E. coli K12.

The fertility of F+ cultures has been attributed to Hfr mutations (205, 211, 214), although other explanations have been suggested (245). Conjugation occurs with high frequency in F-(X)F+ crosses but, unlike Hfr strains which transfer a portion of the chromosome complement containing previously mapped genes, F+ strains in the majority of cases transfer the F agent alone. In selected recombinants, the F agent does not appear to be linked with any known chromosomal markers. On complex media, the F+ property is readily transferred with high efficiency to F- bacteria. From a small inoculum of F+ bacteria, it rapidly spreads throughout an F- population, requiring cell-to-cell contact for its dissemination (245, 247). In minimal medium, the net transfer of F+ is very low, yet virtually all recombinants are F+ (40, 154, 155, 286). F+ transfer precedes in time the transfer of known chromosomal markers (214, 245).

Bacteria genotypically F-, but still infectable to F+, may be taken from F⁺ cultures by use of strongly motile cells (328) or by exposure to acridine dyes (159, 160, 161). Some F+ strains derived by infection with the F agent from other, foreign E. coli cells, readily lose the agent upon growth or storage, yet can retain quite stably F agent from more homologous E. coli strains. As mentioned earlier, F+ bacteria may mutate at various places in the chromosome, forming diverse Hfr derivatives (211, 214, 305). Such chromosomal mutations may be mapped [see (61, 205)] and are generally stable. Both of these findings are also presumed from the differential, but constant, effects on gene transfer (214). Richter (305) has found an Hfr strain (Hfr-3), simultaneously discerned to be mutant at a closely linked mal (maltose) gene, Hfr-3 is unstable both in its fertility and ability to infect recipient bacteria with the F agent. It yields, when crossed with F-, four types of recombinants: (a) F+; (b) Hfr-3; (c) F- which, when infected by F+, becomes F+; and (d) a final rare class which can be selected for by use of the mal marker: F which, when infected by F+, becomes Hfr-3 (305). When Hfr-3 is mated with HfrH, it produces among specific classes of recombinants some F⁻ strains and some compound Hfr's which transfer as HfrH but which segregate Hfr-3 types (306).

The above-mentioned results indicate that specific Hfr mutational sites may exist in two forms, active and inactive, depending upon the presence there of the F agent [Richter (305, 306)]. It has been suggested that the F agent is extrachromosomal but capable of fixation at specific places on the chromosome (214, 216, 245). Lederberg (245) suggests that fertility of F+ cultures, in addition to their content of Hfr mutants, may be brought about by very transient changes in the "quality or position" of the F agent they carry. Such elements which, when present, are capable either of autonomous behavior or of integration into the genome, are termed "episomes" by Jacob & Wollman (214, 216). They apply the term to a general class of conceptually similar phenomena in bacteria and higher organisms. Examination of the literature shows that the term "episome" has been previously utilized with similar mechanistic and structural connotations to explain mutations at the peculiar Bar region in Drosophila (351). These mutations have been more recently attributed to chromosomal aberrations (343), although the means by which the mutant phenotype is engendered still remain obscure. Thompson pictures the gene as "a main particle firmly anchored in the chromosome with varying numbers of one or more kinds of other particles attached. The main particle is called the protosome and the attached particles the episomes" (351), Can a clear-cut distinction be made between "permanently" extrachromosomal factors [plasmids (242a)], "episomes," and the bulwark of classical genetics, the stable chromosomal factor or gene?

The interaction of F agents with bacteria of diverse origin is complex and the agents appear to be capable of undergoing recombination with elements of some bacteria or of being modified by particular hosts. Lederberg & Lederberg (247) and Bernstein (40) have pointed out the diverse behaviors of different F agents in various *E. coli* strains [also see (246, 249)]. Their observations pose a multitude of interesting questions for futher research.

A few recent experiments, utilizing conjugation for genetic transfer, have given valuable insight into certain additional areas of bacterial genetics. They will be briefly mentioned here without any attempt at complete coverage of the entire genome; the reader is referred elsewhere for a thumbnail sketch (149). Markers conferring motility are transferred from *E. coli* K12 into the stably nonmotile, flagellaless *E. coli* B strain (116). One-step streptomycin resistance behaves as a unit character in *E. coli* K12 crosses. Polygenic inheritance is demonstrable in bacterial crosses involving mutants obtained in several steps on increasing concentrations of streptomycin (365).

Lederberg & St. Clair (243, 248) and Hagiwara (136) have reported that $E.\ coli\ F^+$ and Hfr protoplasts are able to undergo conjugation with F^-

bacteria.

Hayes (156, 157, 382) notes that alleles conferring resistance to valine and to azide are dominant and expressed very shortly after penetration into the recipient bacteria. Resistance to T1 phage, previously indicated as a recessive character (242), is delayed in its expression. These studies, and those of Pardee *et al.* (296), indicate that the input genes function almost immediately after, if not during their penetration into the recipient bacteria (i.e., they function much before integration is achieved).

Studies on zygotic induction by Jacob & Wollman (206, 213, 214, 215) suggest that the immune state is a specific active metabolic state of the bacterium, dependent upon extrachromosomal constituents initiated by the prophage. This is inferred from the fact that zygotic induction occurs only when the prophage is transferred from a lysogenic donor into a sensitive recipient and not under the reverse situation, although the critical genes present in the ex-conjugants may be alike in both instances. The effects of ultraviolet irradiation, eliciting induction of prophage, are partially transferred from lysogenic or nonlysogenic bacteria to lysogenic recipients (46). During conjugation there is no transfer of vegetative phage (212) or β -galactosidase [(296); Cohn, Jacob & Wollman, cited in (381)]. We do not know if any other extrachromosomal materials are transferred or not. Specific labelling experiments, as well as thorough examination of the peculiar non-recombinant segregant clones noted by Anderson (10), might be useful in an analysis of this issue.

Based on conjugation studies, Pardee et al. (296) report that the allele which supplies the ability to be induced to form β -galactosidase (lac, i) is dominant to the allele conferring constitutive production of the enzyme. The lac, i locus is a distinct locus, separable in function and by recombination from two loci located at each side of it. These two loci control the specificity of β -galactosidase protein and galactoside-permease, respectively. The lac, i locus acts as a regulating element, affecting both of the other loci in the same manner (inducible or constitutive) [reviewed in (66, 149, 205, 296, 308)].

Holloway's continued studies on recombination in *Pseudomonas* have uncovered behavior in genetic transfer and mating type determination analogous to the system existing in the enterics (163, 164). Hamon (139) observed transfer of streptomycin resistance and bacteriocinogenesis in *Pseudomonas* strains. Since phage-mediated genetic transfer may also occur in *Pseudomonas* (261), the observations reported by Hamon require further clarification as to transfer mechanism. His report of the transfer of bacteriocinogenesis is of interest since, because of the behavior in gene transfer during conjugation in *E. coli*, this would seem to be the most sensitive means of detecting rare bacteria able to undergo conjugation.

BACTERIOCINS AND BACTERIOCINOGENESIS

Bacteriocins have been variously named according to the bacteria from which they are generated (e.g., "colicin" from *E. coli*, "pestin" from *Pasteurella pestis*, etc.). *Mycobacteria* are the most recent addition to the list of bacteriocinogenic genera (275). We will lump them all collectively under the term: bacteriocin.

Bacteriocins are polypeptide or protein molecules, often weakly antigenic, which, when adsorbed to specific receptors on sensitive bacteria, kill the cell. Bacteria are resistant to particular classes of these molecules if they lack the specific sites required for adsorption. Loss of ability to adsorb is controlled by chromosomal factors. Bacteria with the hereditary potential to engage in the synthesis of a bacteriocin are sometimes killed by adsorption of this bacteriocin, released by other cells in the population, but often are partially immune. A number of extensive reviews on bacteriocins, covering the above features, have been written by Fredericq (89, 93, 102, 103, 104, 106, 107) and others (2, 149, 268).

Comparative studies on bacteriocins other than those released by the enterics have only recently been reported. From 23 of 24 Pasteurella pestis strains, Ben-Gurion & Hertman (38) have recovered bacteriocins which appear uniform in all properties tested; all hosts are inducible and release bac-

teriocin only in complex media.

Ivánovics and his co-workers (194 to 201) and Koziński & Pietrzykowska (233, 234) have studied bacteriocins released from Bacillus megaterium strains. Of 200 strains examined, 92 produced bacteriocin. Vegetative cells of all bacteriocinogenic strains tested were uniformly inducible, lysing with the release of bacteriocin only in complex media (201). Koziński also achieved effective induction by irradiation of the medium (234). Some strains also spontaneously released detectable bacteriocin. The lysis after induction was not generally accompanied by release of free phage, as discerned by some (not-too-convincing) electron micrographs or by plating on several indicator strains (less than 1 particle per 109 induced bacteria). Two bacteriocinogenic strains, however, did release a small number of phage particles after induction.

Several rough colony bacterial mutants (192, 193) are more sensitive to killing by B. megaterium bacteriocins than are parent strains. In addition, they are resistant to several phages. The bacteriocins also act upon some strains of B. anthracis, B. subtilis, and some micrococci. Specific receptor sites have not been demonstrated as necessary for their activity. Suspensions of sensitive bacteria are killed by the bacteriocins but show only a very gradual drop in turbidity and no lysis is detected by phase contrast microscopy, although considerable ultraviolet-adsorbing material and β -galactosidase activity is released from them. "Protoplasts" of B. megaterium and E. coli are lysed by the bacteriocins, suggesting a lipaselike molecule which breaks down the osmotic barrier, the cytoplasmic membrane (199), analogous to earlier observations of the effects on protoplasts of a liapse preparation (238, 331). Nonbacteriocinogenic clones may be obtained from spore platings of some bacteriogenic strains.

Of two nonbacteriocinogenic "mutants" derived from a strain which is also an inducible lysogenic, one is nonlysogenic and the second still lysogenic but noninducible (201). These facts suggest an interrelationship between particular carried phages and bacteriocins. Comparative studies with normal and defective lysogenics (172, 268, 270) could be valuable in analyzing the genetics of bacteriocin production. In addition, transformation reactions may soon be available for genetic studies of *Bacillus* species (333). It must be realized that the "bactericins" of *B. megaterium* are but one of many types of possible lethal agents, quite different in their origins and modes of action.

Bacteriocins of enteric organisms are classified by their host specificities in adsorption and killing, each class receiving a capital letter designation (e.g., B, E, I, K, V, etc.) [reviewed in Fredericq (89, 93, 103)]. Some receptors are shared in common with certain bacteriophages [reviewed in (90, 91, 93, 103, 149)]. Bacteriocins may be used for bacterial typing [(e.g., Hamon (143)]. One striking case has been noted by Fredericq (90, 91) in which spontaneous mutants of E. coli, bacteriocinogenic for bacteriocin V, mutated to simultaneous resistance to phages T1, T5, and T7, to bacteriocin C, and also produced bacteriocin M, a new bacteriocin not detected as produced by the wildtype strain. A second instance of a similar nature is provided by Cocito & Bryson (64) who found that T3-resistant mutants of E. coli B produce a bacteriocin to which the parental B strain is sensitive. Fredericq had previously noted relationships between antigenic structure and bacteriocin production [e.g. (88)], but these leads have not been pursued. Older reports in the literature [e.g. (290)] indicate simultaneous changes in probable bacteriocin production, growth characteristics, and fermentation abilities. While these early studies are impossible to interpret in modern terms, such associated changes should be searched for in the future.

Bacteriocins within a class, such as E, have been subgrouped according to the specific immunity bacteriocinogenesis sometimes confers upon the cell [e.g., E1 and E2 show distinct immunity-conferring patterns, yet utilize a common receptor site (100, 102, 103, 109)]. The genetic factor eliciting a bacteriocin may be lost from the cell with a simultaneous loss of immunity, Immunity, however, is often only partial, (100, 102, 141). Some bacteriocinogenic strains are completely sensitive to the bacteriocin they themselves produce under special conditions of culture or after induction; they are able to mutate to resistance by loss of the specific receptor (317). Adsorption on sensitive bacteria of certain bacteriocins specifically excludes the multiplication of certain phages (93, 94). Synthesis of bacteriocins sometimes interferes with phage-production after induction of bacteria which are simultaneously bacteriocinogenic and lysogenic (97, 141). This behavior may result in changes in the phage typing reaction although detectable serological differences are absent (141). Bacteriocinogenic strains which are also lysogenic show a positive correlation in the ability to produce bacteriocin B and the inducible phages of Fredericq's type II (92).

Transfer of bacteriocinogenesis has been found to occur between a number of enteric "species" and "genera" [(95, 98, 140, 141, 144), Fredericq, in (73)], including transfer to *E. coli* from supposedly F- organisms such as Salmonella and Shigella (73, 140). The transfer has now been demonstrated to be mediated by two different means: (a) conjugation (discussed below),

and (b) generalized transduction [Fredericq in (73)]. Fredericq has obtained derivatives of Salmonella typhimurium strain LT-2 bacteriocinogenic for bacteriocins E, I, and K by mixed growth with various bacteriocinogenic enteric species. When bacteriogenic for K, strain LT-2 is no longer sensitive to P22 phage. Ability to produce bacteriocin E2 could be transduced at low frequency with P22 phage (one transduction per 106 phage); the ability of the phage to transduce str-r or prototrophic markers was unaffected. Derivatives of E. coli K12, bacteriocinogenic for E1 E2, V, B, K, or I, served as effective donors for the transfer by P1 phage of bacteriocinogenesis for E1 and B. No transfer of E2 could be demonstrated. Further, the ability of P1 phage to transduce prototrophic markers was much reduced or was abolished when grown on the bacteriocinogenic strains; this was observed in spite of the absence of demonstrable bacteriocins in the phage preparations and when

bacteriocin-resistant mutants were used as recipients.

Transfer of a number of bacteriocinogenic factors during cell conjugation has been demonstrated by Fredericq [reviewed in (102 to 107, 149)]. In certain E. coli K12 crosses, bacteriocinogenesis for E1: (a) is transferred from the donor with high frequency; (b) appears early in the F-bacteria; (c) does not appear to be linked with any of the few chromosomal markers tested; and (d) does not affect "appreciably" the fertility of the cross. All recombinants are bacteriocinogenic in the reciprocal cross [Frederica (95, 96, 99, 105, 109)], Alfoldi et al. (5, 6) using resistant mutants as test organisms, have presented a precise analysis of the activity of the bacteriocinogenic factor derived from E. coli strain K30 (which appears to be the same as that variously designated by Fredericq as "E(1)" or "ER" and mentioned above simply as E1). Alfoldi and his co-workers found that transfer of (E1)—the parenthesis and italics are utilzed as the symbol for the bacteriocinogenic factor-begins in 3 to 4 min, after mixing of bacteriocinogenic F+ or HfrH with nonbacteriocinogenic F- cells, in confirmation of Fredericq's earlier pronouncements. Transmission to 80 per cent of the F population is achieved in 1 hr. The proportion of bacteriocinogenic recombinants is the same for two marked regions. Alfoldi et al. (6) suggest that the bacteriocinogenic factor is transiently induced upon penetration into the F- bacteria and multiplies, only to fix again at a specific site on the chromosomes of the progeny from the F ex-conjugant. In the reverse cross, that is, $F^-(E1) \times Hfr(nonbacteriocino$ genic), there is no transfer of (E1) to the Hfr. Most interesting is the finding that about 90 per cent of the bacteriocinogenic hosts die. Furthermore, nonbacteriocinogenic bacteria are never found among selected recombinants. Utilizing this lethal zygosis as a "marker," Alfoldi and his co-workers (6) have located the allele of (EI) on the bacterial chromosome, near the site of origin in the HfrH strain. Besides (E1), Frederica notes a second factor. (S₂), which is also transferred with high frequency (95). Fredericq describes E. coli K12 derivatives which carry (E1) or (B) and, simultaneously, are lysogenic for \(\lambda\); upon induction, these bacteria lyse with the release of normal bursts of λ and normal production of bacteriocin (97, 98, 99).

Bacteriocinogenesis for B, K, and E2 only slightly reduces fertility when the F- strain carries the bacteriocinogenic trait. The relative proportions of recombinants for a restricted number of chromosomal markers tested has been reported as unaffected by the presence of bacteriocinogenic factors in the recipients; all recombinants remain bacteriocinogenic. Suppression of fertility is complete if the F+ strain is the bacteriocinogenic partner; in this case it is assumed that lethal synthesis of bacteriocin begins when it penetrates into the F- cell (105, 110), analogous to zygotic induction of inducible prophage. This last proposition is amenable to direct test and the former finding is liable to closer scrutiny in tests with a suitable array of wellmarked Hfr strains. An earlier report (95) described both the transfer of (B) and (K) and recovery of recombinants in a frequency which would tend to rule out phage-mediated transduction. Evaluation of these systems, thus, must await publication of more complete data. Induction of E. coli K12 lysogenic for λ and bacteriocinogenic for E2 or K, results in slightly premature lysis with the release of bacteriocin and a greatly reduced burst of λ (97).

By studying the dependence of bacteriocin activity on concentration, Jacob et al. (203, 209, 210) have deduced that single particles of bacteriocins can kill sensitive bacteria. The adsorption of bacteriocins inhibits many metabolic functions, but the mechanisms of action are not known. There are no reports that bactericins have been examined for enzymatic activity or even for their cytological effects on sensitive hosts. Lederberg & St. Clair (248) have found that bacteriocins E, K, and V exert only slight activity on protoplasts and L-forms of E. coli.

Fredericq (88) noted that there is a relationship between the antigenic structure of bacteria and their ability to produce a specific bacteriocin. The only extensive biochemical study reported to date is that by Goebel and his colleagues on bacteriocin K (7, 34, 35, 120 to 123). Bacteriocin K is a protein, closely bound to a lipopolysaccharide complex which consititutes a somatic antigen of the bacterium.

Cell-to-cell transfer of bacteriocinogenesis for bacteriocins V and I has been discerned only rarely [(97, 101, 105); Fredericq in (73)]. When (I) is present in the recipient bacteria and certain markers of the F^+ parent are utilized to select for recombinants, the cross is sterile (105).

BASIC RESEARCH AND SOME PRACTICAL PROBLEMS

It is highly likely that cell-to-cell transfer of genetic materials occurs frequently in mixed populations of bacteria in nature. Recent reports (19, 77, 322) may be added to older descriptions of the isolation of two or three different "species" of Salmonella during a single outbreak or even from the same patient. The various means of genetic transfer allow these, presumably haploid vegetatively reproducing, opportunists to take advantage of special conditions for rapid proliferation, in which a large group of relatively isogenic descendants may be formed. Where a change is desirable, however, mutation-selection and internal genetic controls can be critically supplemented by re-

combination of beneficial traits. Basic research in bacterial genetics offers, along with adequate analysis at the enzymological (not just the descriptive) level, an understanding of bacterial variation, so important in the pathogenicity and economic usefulness of various bacterial species.

It should be obvious from the data surveyed in this review, as already pointed out by bacterial geneticists elsewhere [for example (263, 266, 324, 338)] the present classification of bacteria but little reflects the actual evolutionary status and relationships between species (used in any of the several more classical interpretations of the word). A "slow" and progressive modification of existing nomenclature, as suggested by some [(349); others] would seem unnecessarily disruptive; it hardly seems that a constantly fluctuating systematics, even if it is headed in the right direction, is a solution. Perhaps it would be best to let taxonomy rest for awhile until a truly knowledgeable solution is formulated, which can stand the test of time. Indications abound for close genetic interrelationships among bacteria, which are, as yet only, transiently investigated [e.g., data on phage hosts in (332, 345), etc.]. The data surveyed in this review on antigen conversions indicate that even some of the newer hopes of the taxonomist, for example, the chemical analysis of the insoluble and nondigestable residues of bacterial cell walls, will not offer much of a solution except in extreme ranges. Data on DNA composition of bacteria (37, 251, 366) offer a theoretically pleasing approach but fail in their sensitivity to discern small differences in base ratios. The ultimate solution to an enduring and stable taxonomic system would appear to reside only in thorough analysis at the genetic level; there is no reason to believe that such analysis is not possible in any group of bacteria if looked for thoroughly and with proper techniques.

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SULPHATE REDUCTION BY BACTERIA¹

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Though many bacteria reduce sulphate during the synthesis of sulphurcontaining amino acids, this review will be restricted to the dissimilatory sulphate-reducing bacteria: those whose major energy-yielding reactions are linked to a reduction of the sulphate to the sulphide ion. By analogy to Kluyver's classification of bacterial nitrate reductions (73), the smaller-scale reductions of sulphate to sulphur-containing amino acids are best referred to as "assimilatory" sulphate reductions, even though (a) sulphide may appear transiently as an intermediate, and (b) death and autolysis of the cell may lead to the ultimate release of sulphur again as H₂S [see (26)].

The dissimilatory sulphate-reducing bacteria have not been the subject of a comprehensive review since 1936 (18), and the sparseness of bibliographies in recent papers about them, together with the frequency with which known or dubious observations are reported as new, indicates a widespread ignorance of the published material even among workers handling these bacteria. A comprehensive review is clearly overdue. This, however, is impracticable within the confines of the present article and the writer will therefore concentrate on certain topics connected with the bacteria which seem to him to be of immediate interest; important omissions of which he is aware include their nutrition, hydrogen metabolism, chemical composition, inhibition, and details of their economic activities. Reviews of limited aspects of their physiology or economic functions have been given by Starkey (129, 130, 131); Updegraff (139); Beerstecher (16); Butlin & Postgate (23, 26); Postgate (104), and ZoBell (144).

CLASSIFICATION

The belief that dissimilatory sulphate reduction was the property of a wide variety of bacteria was rightly criticized by Baars (11) who demonstrated that it was, in fact, a property of very few species. He suggested that the thermophilic and marine sulphate-reducing bacteria were variants of the fresh-water species now called Desulfovibrio3 desulfuricans; on the basis of their ability to use various carbon sources he distinguished two other species: D. rubentschikii, able to utilize acetate, butyrate, or propionate, and D. rubentschikii var. anomalous, able to utilize butryate or propionate but not acetate. D. desulfuricans did not metabolize any of the lower fatty acids (except

With two exceptions, the survey of the literature pertaining to this review was concluded in September, 1958.

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³ In this article the generic name Desulfovibrio is used; earlier synonyms are Spirillum, Microspira, Vibrio, and Sporovibrio.

formate) and, indeed, formed acetate as an end product from most C sources which were more complex than C2, though primary alcohols such as butanol yielded the corresponding aliphatic acid. ZoBell & Rittenberg (146) preferred to regard the marine type as a separate species; Littlewood & Postgate (78) determined the distribution of salt-sensitive and salt-tolerant variants in marine and fresh-water strains, and favoured Baars' view on the taxonomic question though their experiments confirmed those of both parties. Attempts to reisolate D. rubentschikii in the present author's laboratory have met with complete failure (33), though mixed populations able to metabolize butyrate or acetate have readily been obtained. The ubiquity of contaminant obligate anaerobes (as well as other types) in enrichment cultures, as well as the difficulty of detecting such contaminants (100) opens the possibility that earlier cultures of D. rubentschikii were impure. In the author's opinion, these are grounds for reasonable doubt whether strains of sulphate-reducing bacteria which are able to utilize acetate, propionate, or butyrate, exist.

Baars's view that thermophilic sulphate-reducing bacteria—organisms which demonstrably formed spores and were multiflagellate—were variants of the nonsporulating and uniflagellate mesophile was supported by Starkey's experiments (128) indicating that the two strains could be interconverted by "training." These experiments must have been wrongly interpreted. Campbell (28) and others have identified the sporulating thermophile unequivocally as Clostridium nigrificans, using Starkey's strains among others, and thus supported the contention of Prévot (105) that sulphate reduction occurs among the clostridia. Experiments in the present author's laboratory fully confirm those of Campbell et al., and a critical reading of the early descriptions by Elion (39) and Baars (11) indicates that the original thermophiles were probably also C. nigrificans. Consequently, it seems likely that a truly thermophilic Desulfovibrio desulfuricans has never been observed; heat-resistant strains have been obtained in the laboratory by training procedures but their temperature maxima lie below 50°C.

Hvid-Hansen's Desulphoristella (50) has been lost, and his description did not provide satisfactory evidence that it was a single pure species. ZoBell & Morita (145) reported a barophilic sulphate-reducing bacterium of unusual morphology; a new multiflagellate sulphate-reducing mesophile D. orientis has been reported (2) which lacks certain pigments characteristic of D. desulfuricans and occasionally forms spores. The only species of sulphate-reducing bacteria available from recognized collections of type cultures at present are D. desulfuricans, its aestuarii variety, D. orientis and C. nigrificans. Cowan's recommendation (35) that new organisms should be deposited in recognized collections applies with particular force to a group so subject to taxonomic reconsideration.

CULTIVATION

Early recipes for culture media (11, 128) included a considerable concentration of ferrous iron salts to act as an indicator of growth by blackening

caused by FeS. These large amounts of ferrous ions are unnecessary, but traces of iron are required by *D. desulfuricans* (22). Growth of *D. desulfuricans*, *D. orientis*, and *C. nigrificans* is increased by the addition of yeast extract to the culture medium (2, 22); improved growth of *D. desulfuricans* in a medium of peptone, yeast extract and glucose, (96) was attributable to use of an impure culture (100). Miller recorded media, some of which included organic supplements, which permitted high yields of H₂S (84, 85).

The mesophilic sulphate-reducing bacteria are more exacting in their requirements for anaerobic conditions than are most anaerobes. They require an E_h in the neighbourhood of -200 my, for the initiation of growth, and inattention to this detail probably accounts for their reputation for being "difficult" organisms to grow and isolate, Many of the precautions reviewed by McClung (82) for the cultivation of clostridia are insufficient when applied without modification to sulphate-reducing bacteria. Cysteine or Na₂S (44) accelerates growth by lowering the E_h sufficiently; reducing agents of more oxidizing E_0' values, such as ascorbate or ferrous ions, are somtimes but not always effective (45). Colony counts or dilution counts in fluid media are valueless unless the E_h is controlled (44); the use of cysteine has provided a valid procedure for obtaining colony counts with pure cultures (45); with natural samples the cysteine procedure may be used only with fluid media (38, 45) as all positive tests must be checked for cysteine-decomposing bacteria. A promising agar medium has been reported as suitable for natural samples, having its E_h poised with both ascorbate and thioglycollate (6). It may be used with membrane filters and a "scavenging" aerobe (138) but, unfortunately, neither of these reports cites recovery data with known populations. Abd-el-Malek & Rizk (1) found counts comparable to those given by the cysteine procedure using liquid media containing metallic iron as a reducing agent; their account did not include tests with a pure strain, but unpublished experiments (Abd-el-Malek, personal communication) indicate that an excess of nonsulphate-reducing bacteria is not essential for the success of their method. Dostalek and his colleages (37, 126) abandoned conventional dilution procedures for estimating these bacteria in oil well waters and obtained nomograms relating the microscopic count of D. desulfuricans to the rate of production of H2S in impure culture. They then used the rate of H₂S formation after inoculation into a culture medium to estimate the numbers of sulphate-reducing bacteria in samples of oil well waters.

Several vagaries in the behaviour of sulphate-reducing bacteria. such as their enhanced resistance to inhibitors in the presence of ferrous ions, the tendency for their colonies to grow in association, the inactivity of certain nutrient amino acids in the absence of cysteine, etc., can be explained in terms of the requirement for a low E_h (44). The phenomenon of "skipping," in which a soil sample, for example, will show growth of sulphate-reducing bacteria with a 0.1 gm. portion but none with 1 gm., can be explained similarly if the sample is regarded as containing materials poising the E_h in a relatively oxidizing range.

The isolation of pure cultures may be much facilitated by the use of media of poised E_h ; stringent tests for the presence of contaminant obligate anaerobes, as well as faculative aerobes, are desirable before a culture is accepted as pure (100).

The availability of valid methods for counting these bacteria will allow, for the first time, a quantitative study of their ecology, particularly in relation to corrosion, pollution, sulphur-formation, and other phenomena of economic importance to be discussed later. A start has been made with an ecological survey of Hiroshima Bay (67) and an estuarine environment in Japan (69, 70), using a medium poised with ascorbate. The present writer has not found media of E_h poised solely with ascorbate satisfactory, but the published survey indicates that they gave quantitative recoveries in this instance. Senez et al. (118) studied the populations of polluted gas holder waters using cysteine; a polluted clay pit (38) and a brackish lake (41, 41a) have also been studied quantitatively.

CARBON METABOLISM OF D. DESULFURICANS

This organism has been at various times described as able to metabolize carbohydrates, petroleum hydrocarbons, long-chain fatty acids, alcohols; equally frequently this has been denied. A catalogue of references would be wearisome; it is more constructive to point out the possible sources of error which should be allowed for in considering published findings: (a) Authentic pure cultures have not always been used; for example, a published report exists of D. desulfuricans A.T.C.C. 7757, utilizing acetate and forming gas from glucose, (which, by definition and, in fact, it does not) and must be attributed to infection of the stock; purification of an infected strain is known to reduce its range of fermentative ability (68). Explicit checks for contaminant anaerobes are most frequently absent from published data; tests for contaminants using Petri dishes, even in anaerobic environments, are inadequate (100). (b) The presence of yeast extract in the culture medium influences the apparent ability of strains to utilize diverse carbon sources: Pochon & Chalvignac (95) obtained a strain, maintained in pyruvate media, which they claimed required yeast extract for growth in lactate media of unstated E_h . (c) The E_h of the medium influences the apparent "spectrum" of compounds attacked (44); the wide range of substances attacked by Baars's (11) strains compared with the small number of substrates reported by subsequent workers may be attributed to his routine practice of adding ca. 15 p.p.m. sterile H_2S water to his cultures, thus lowering the E_h to ca. -200 mv. This admirable practice has been neglected by most other workers but is essential for critical studies of this kind. (d) The purity of more unusual reagents such as hydrocarbons should be such that, at the concentration tested, there is no possibility of marginal growth on impurities. Critical data on this point are often absent from published work.

The reputed pleuripotency of *D. desulfuricans* should therefore be regarded with some reserve, but the terminal metabolism of simple 3- and 4-carbon compounds by marine strains is on a sounder basis and has been

studied biochemically to some extent. Lactate oxidation to acetate proceeds by way of pyruvate (46, 110); the latter substrate is interesting in that, with certain strains, it supports growth without concomitant sulphate reduction (99). A report (110) that pyruvate in the presence of sulphate permitted twice as much growth per mole substrate as did lactate, was mistaken (Dr. J. Senez, personal communication). Cell suspensions metabolize pyruvate to acetate, CO₂, and free H₂ in the absence of sulphate (112); participation of a formic hydrogenlyase has been proposed (58). Cell-free enzyme preparations have been obtained from fresh-water strains able to dismute pyruvate to acetyl phosphate, CO₂, and either H₂ (87) or ethanol (108).

A salt-water strain dismuted malate, via fumarate, to succinate and acetate in the absence of sulphate; in its presence succinate was further oxidized to acetate (46). These observations suggest the existence of a terminal cycle comparable to the tricarboxylic acid cycle [see references (26, 104) for brief discussions] but evidence for the complete cycle is lacking; the absence of dissimilatory acetate metabolism suggests that some features of the conventional cycle may be lacking, and that terminal metabolism of certain Acetobacter spp., in which the cycle is reputedly absent (71), may provide a closer analogy. The broad analogy of the behaviour of these bacteria to that of the acetic acid bacteria was pointed out by Kluyver (72) and has been enlarged upon in a recent review (104).

Autotrophic growth-by which is meant growth at the expense of exclusively inorganic substrates—was observed by Starkey & Wight (131), using impure cultures in media containing mineral salts, sulphate, and bicarbonate in an atmosphere of H2. Butlin & Adams (21, 22) confirmed their observations using pure cultures. Materials of conunercial "Analytical" grade and conductivity water were used in preparing the mineral media; neither of the precautions, in the writer's opinion, entirely eliminates the possibility that apparently autotrophic growth took place at the expense of marginal organic impurities in the medium. Sisler & ZoBell's experiments (120), demonstrating a quantitative increase in organic carbon in "autotrophic" cultures, would appear to clarify the point, but they regrettably published no quantitative details. In the writer's opinion, there remains the possibility that these bacteria fall more properly into Lwoff's (79) nutritional category of metatrophs: organisms capable of growth at the expense of CO2reduction but requiring small amounts of organic nutrients. Using C14, Sorokin (122) demonstrated CO2 fixation during sulphate reduction in hydrogen; these processes could take place successively, sulphate reduction involving uptake of inorganic phosphate and CO2-fixation involving its release. Thus, the concept of separation in time of assimilation and dissimilation, postulated by Vogler & Umbreit for Thiobacillus and disputed by the Baalsruds [see Vishniac & Santer (142) for a resumé of this question], has been revived for Desulfovibrio. Sorokin (123) has also studied growth in putatively autotrophic conditions and reported that 10 to 20 gm.-ions of SO4" were reduced for each mole of CO2 fixed. In terms of free energy expended per mole of CO2 fixed, this finding is comparable with those for other autotrophs (104); as Sorokin (124) has pointed out, the thermodynamic "efficiencies" that have been used in discussion of chemoautotrophy (10, 91) have little meaning when the assimilatory reaction

$$CO_2 + 2H_2 \rightarrow [CH_2O] + H_2O$$

involves a free energy change of approximately zero. The fact that the reduction of CO₂ by hydrogen to the carbohydrate level is energetically neutral or slightly endergonic (104), makes one view with surprise Sisler & ZoBell's (120) success in growing marine *D. desulfuricans* in mineral media with H₂ and bicarbonate but without detectable sulphate.

The pathway of CO₂ assimilation and its relationship to Calvin's cycle for photosynthesis in green plants and other autotrophs present obvious possibilities for research, but have not so far been the subject of published work

TERMINAL ELECTRON TRANSPORT IN D. DESULFURICANS

D. desulfuricans is moderately rich in flavoproteins which possess an uncommonly high ratio of flavin-adenine-dinucleotide to the mononucleotide (93). In addition, this species contains a cytochrome now referred to as c_3 , among the first to be reported in an obligate anaerobe (23). This has been the subject of numerous papers by Ishimoto and his colleagues (51 to 55, 57, 59, 60) whose findings agree essentially with those reported by the writer (101); cytochrome- c_3 is a basic (pH ca. 10.5) bifunctional haematohaematin of low potential ($E_0' = -204$ mv.) and of molecular weight ca. 13,000 (Fe content ca. 0.9 per cent). The reduced α -peak lies at 553 m μ ; the protein is stable to heat and acids, has thio-ether linkages between the two haematin residues and the apo-protein, is water soluble and may easily be obtained in a purity of at least 94 per cent by ion exchange chromatography. It is accompanied in the cell by a porphyro-protein of unknown function which has been called desulfoviridin.

A physiological function for cytochrome- c_3 has been indicated in the metabolism of the following substrates:

Sulphate.—Partial oxidation of intracellular c_3 occurs when cells are incubated with sulphate; the reaction is delayed by selenate or monofluorophosphate⁴ (101) which are known competitive inhibitors of the sulphate reductase system (98). Bacteria deficient in c_3 obtained by culture in iron-deficient pyruvate media (102) have a markedly reduced ability to reduce sulphate. These are the only direct lines of evidence which indicate that c_3 is concerned in sulphate reduction; the question has recently been discussed critically (26). Enzyme preparations able to reduce sulphate have not been obtained, so enzymological evidence on the function of c_3 in sulphate reduction is not available.

Thiosulphate.—Among the first reductases to be extracted from D.

⁴ The ion PO₃F = is a structural analogue of SO₄ =, not of PO₃ =, and has the enzymyological properties of a sulphate antagonist. It is interesting that Rapp & Slewinski (106), believing it to be a phosphate analogue, succeeded in observing competitive antiphosphate activity in phosphorylase preparations.

desulfuricans was the soluble thiosulphate reductase (56); Ishimoto and his colleagues showed that this enzyme, with hydrogenase and a viologen dye as carrier, reduced thiosulphate to sulphite and sulphide; they later observed that cytochrome- c_3 would replace the viologen dye (51, 59), and partially separated the thiosulphate reductase from hydrogenase c_3 , and desulfoviridin (52). Ishimoto's group have reported the effect of various inhibitors on the thiosulphate reductase system (60).

Tetrathionate.—Crude thiosulphate reductase preparations sometimes reduce tetrathionate (101). When a viologen dye is present, this is a non-enzymic process arising from spontaneous reaction between reduced viologen and tetrathionate; when c_3 is the carrier, however, the reaction is enzymic

because tetrathionate does not oxidize ferrocytochrome-ca (60).

Sulphite.—Crude extracts of vacuum-dried bacteria contain a sulphite reductase (88); particulate enzyme preparations are more active (104). The activity of sulphite reductase preparations is much stimulated by viologen dyes; cytochrome- c_3 was reported to have a modest stimulating effect (101), though Ishimoto's group could not confirm this (60); nevertheless, reversible oxidation of c_3 by sulphite can readily be observed spectroscopically with intact cells. Unpublished work in the writer's laboratory indicates that particulate sulphate reductase preparations require at least two cofactors in addition to c_3 ; these are soluble, thermo-labile proteins.

Oxygen.—Cytochrome-dependent reduction of oxygen can be demonstrated with heavy suspensions of sulphate-reducing bacteria (101) in spite of their exigent anaerobic habit. This reaction is of no biological importance to the organism; it involves a nonenzymic reaction between c₃ and oxygen and

is a consequence of the low standard potential of c_3 .

Hydroxylamine.—The kinetics of hydroxylamine reduction by sulphatereducing bacteria are those of a typically enzymic reaction, involving penetration to the reaction site in one strain but not in a second (115, 116). Cytochrome- ϵ_3 or a viologen dye act as carriers, but it is now clear that the oxidative step is nonenzymic; the organism possesses no specialized hydroxylamine reductase (57, 116, 117).

Nitrite.—Though usually unable to utilize nitrate as N-source, these bacteria reduce nitrite vigorously in hydrogen (119). This reaction is also cytochrome-c₃-dependent, and work in the author's, Senez's, and Ishimoto's

laboratories indicates that the terminal stage is nonenzymic.

Colloidal sulphur.—Sulphate-reducing bacteria do not reduce native sulphur unless the particle size is small (97); Ishimoto's group have shown that this, too, is partly a nonenzymic reaction (57, 60) depending on the chemical oxidation of ferrocytochrome- ϵ_0 by colloidal sulphur.

Formate.—Cell-free extracts of D, desulfuricans have little formic hydrogenlyase activity, but the addition of c_3 or a viologen permits the evolution of H_2 and CO_2 from formate (57). The action of c_3 in this respect recalls the factor " x_2 " of Gest & Peck (42), which is needed to link a clostridal hydrogenase to a formic dehydrogenase from a coliform organism; this, too, was replaceable by a viologen.

Hydrogen ion.—In the presence of an excess of sodium dithionite, together with methyl viologen as a carrier, hydrogenase preparations act in "reverse":

$$MV^+ + H^+ \rightarrow MV^{++} + \frac{1}{2}H_2$$
 (92, 135)

Ishimoto's group have shown that cytochrome- c_3 will replace the viologen in this reaction (57).

Pyruvate.—Cytochrome- c_3 acts as a terminal electron acceptor for the pyruvic dehydrogenase system (117). Pyruvate metabolism in these bacteria can also involve a phosphoclasm yielding free H_2 (87, 112) but, in unpublished experiments, Millet was unable to demonstrate any cofactor activity on the part of c_3 with appropriate enzyme preparations. The ability of the organism to metabolize pyruvate when deficient in iron, and hence in c_3 , also suggests that c_3 is not concerned in the phosphoclastic pathway of pyruvate breakdown.

In summary, it is clear that c_3 has the properties of a terminal electron carrier analogous to the cytochrome system of aerobic tissue, in spite of its being a 2-electron transporter, and the assumption that it plays such a physiological role in sulphate reduction is a reasonable working hypothesis. But its participation in a number of reactions that are clearly "biochemical artefacts" (e.g., oxygen, hydroxylamine, or sulphur reduction), raises the question of how many "reductase" activities in other organisms are artificial in the sense that their terminal process is a spontaneous reaction between substrate and an electron carrier of low standard potential; possibilities of this kind were cautiously pointed out by Senez & Pichinoty (117).

D. orientis contains an insoluble cytochrome that is not c_3 (2); no evidence exists for a cytochrome in C. nigrificans, though haematin compounds are apparently present (28, 103).

PATHWAY AND SITE OF SULPHATE REDUCTION IN D. desulfuricans

It is likely in principle that sulphate reduction takes place in stages (11) although, as van Niel wrote in an admirable essay (89), Baars's formulation of the reaction sequence must be taken merely as a first approximation. The variety of oxy-acid anions of intermediate oxidation formed by sulphur, together with their lability and tendency to interact at physiological pH values, makes this subject complex. An up-to-date list of compounds of these reduced by D. desulfuricans is given in Table I; simple inspection of this reduces the potential inorganic intermediates to sulphite, thiosulphate, tetrathionate, and colloidal sulphur. Postgate (97) showed that the kinetics of substrate reduction in H₂ by whole cells were consistent only with sulphite as an intermediate, a view later supported by the isotopic experiments of Koyama et al. (74): sulphite inhibited reduction of labelled sulphate, whereas thiosulphite did not. Millet (88) provided the first unequivocal demon-

⁵ To the polythionates have been added in recent years, the sulphane-monosulphonates (109), thus approximately squaring the "degree of uncertainty" in this field.

TABLE I
THE REDUCTION OF SULFUR COMPOUNDS AND ANALOGUES BY
Desulforibrio desulfuricans

Substrate	Reduction	Comments
SO ₄ "	+	
SO ₃ "	+	established intermediate in SO ₄ " reduction
$S_2O_3^{\prime\prime}$	+	
S ₄ O ₆ "	+	(not reduced by D. orientis)
$S_2O_4^{\prime\prime}$, $S_2O_5^{\prime\prime}$	+	decompose into one or more of the ions above at physiological pH values
$S_3O_6^{\prime\prime}$, $S_5O_6^{\prime\prime}$	_	
Colloidal S	+	partly nonenzymic; see text
Ordinary S	-	if free of oxidized impurities
S_2O_6'', S_2O_8''		
SO ₃ NH ₂ ', SO ₃ Et',		
SO ₃ φ', SO ₂ toluene',	-	
SO ₃ CH ₃ ′, SO ₂ φ′,		
Taurine, cystine, cysteic acid		
O_2	+	see text
SeO ₃ ", SeO ₄ "	±	reduced at subinhibitory levels, since reaction with S" yields Se
CrO4", PO3F", (TeO4"), ClO4"	-	
Methylene blue, tetrazolium, etc	. +	redox dyes and other conventional elec- tron acceptors are usually reduced
Fumarate, malate	+	reduced to succinate in absence of sul- phate
NH ₂ OH, NO ₂ '	+	partly nonenzymic; see text
NO ₃ '	-	see text

stration that sulphite, or something in biochemical equilibrium with it, is an intermediate is normal sulphate reduction, by demonstrating the formation of labelled sulphite from labelled sulphate. Aubert (8) demonstrated that the sulphur entered organic combination at the sulphite level of oxidation, but the nature of the compound formed eluded him. This experiment is the sole published evidence that sulphate reduction might involve an organic pathway.

Experiments with heavy cell suspensions indicate that sulphate does not freely penetrate resting cells of *D. desulfuricans*; moreover, cells which are plasmolyzed by 6 per cent NaCl solutions are uninfluenced by 15 per cent solutions of Na₂SO₄ (77). This paradoxical situation is not easily explained, but it suggests that those bacteria have an osmo-regulatory system especially concerned with sulphate and makes possible the assumption that the osmotic barrier may be the site of sulphate reduction. It is likely from first principles that reduction of a spherically symmetrical divalent ion like

sulphate would require a preliminary endergonic "activation"; the "active sulphate" of arylsulphatase action presents a possible manner of sulphate activation, but no experiments on its reduction by sulphate-reducing bacteria or their extracts have been reported. D. desulfuricans does not possess conventional aryl-sulphatases (33).

NITROGEN METABOLISM

Nitrate is not reduced and often inhibits the growth of sulphate-reducing bacteria (5, 36, 62, 107, 119); the contrary report by Baumann & Denk (13) can reasonably be attributed to contaminant anaerobes in their cultures. Nitrite and hydroxylamine are reduced readily (119), but as discussed earlier, these are "biochemical artefacts" involving no specific reductases. Fixation of gaseous nitrogen has been claimed by Sisler & ZoBell (121) using mass spectrometric procedures; Senez, Le Gall & Pichinoty (personal communication) have recently isolated strains which show N-fixation both with ¹⁵N and by conventional analyses.

The ability of amino acids to serve simultaneously as C and N sources for sulphate-reducing bacteria still requires systematic examination. Five strains of marine D. desulfuricans and one of C. nigrificans studied by Senez (111) gave weak growth with leucine but not six other amino acids as C (but not N) sources; later utilization of cysteine was demonstrated (114). Cell suspensions of a marine strain did not decarboxylate or deaminate 17 amino acids, nor did the glycine-leucine or arginine-glutamate Stickland reactions occur (111); oxidative decarboxylation with sulphate as hydrogen acceptor was not sought. Acetone powders contained a transaminase system capable of conducting the amination of α -ketoglutaric acid at the expense of either aspartic acid or α-alanine. Using a different marine strain, Senez & Cattanéo-Lacombe (113) showed that α-alanine was an intermediate in the first of these reactions, being formed by an ω -decarboxylase from aspartate. The ω-decarboxylase was inhibited by iso-nicotinic acid hydrazide and stimulated competitively by pyridoxal phosphate (29); it has been used for the selective degradation of labelled aspartate (9). A wider range of amino donors for the formation of glutamate has been claimed with a fresh-water strain (4).

Cysteine metabolism by a marine strain yielded acetate, NH₃, H₂S, and CO₂ if sulphate were present; in its absence free H₂ was evolved (114). The reaction in the absence of sulphate recalls Kallio & Porter's (66) preparations from *Proteus*, which consisted of cysteine desulphhydrase coupled to a phosphoclastic pyruvic dismutase. With *D. desulfuricans* the system differed in that azide did not block the reaction at the pyruvate stage.

ECONOMIC ACTIVITIES

Sulphate-reducing bacteria have been implicated in so wide a variety of phenomena which can be broadly classed as of economic importance that they cannot all be discussed in detail here; economic activities involving their autotrophic functions have been briefly reviewed (24). References to

the phenomena are widely scattered, and the writer feels that the following catalogue, in spite of its skeleton bibliography and commentary, is probably the most useful way of providing a preliminary guide to these matters.

Anaerobic corrosion of iron and steel.—This process was mainly attributed to cathodic depolarization by the bacteria through the agency of hydrogenase (129, 131, 139); recently, grounds for revising this view have been reviewed (130).

Acid corrosion of metals and stone.—Sulphide formed by these bacteria, or sulphur compounds generated therefrom, may be oxidized by *Thiobacillus* spp. to H₂SO₄, causing corrosion of stone (94), concrete [see (24)], and metal pipes (31).

Formation of sulphur deposits.—It seems to be agreed that the reductive steps of natural sulphur formation are caused by sulphate-reducing bacteria (24), but some disagreement exists whether the oxidative steps are attributable to photosynthetic bacteria (25, 63), a reaction between CaSO₄ and H₂S yielding sulphur (40, 64), or the action of thiobacilli (61). All three probably operate, depending upon the environment.

Formation of soda deposits.—Owing to the stronger acidity of CO₂ compared with H₂S in water, the final product of sulphate reduction in nature is a carbonate; if Na₂SO₄ is the sulphate source, soda deposits are formed

(18, 141).

Formation of metal sulphide deposits.—A function in this phenomenon, discussed by Starkey & Wight (131), has been supported by Miller (86).

Formation and release of natural oil deposits.—The position in this field was briefly reviewed by Stone & ZoBell (132), and more extensively by Appert (7) and by Beerstecher (16); the situation is nevertheless confused, and simple contradiction of evidence from different laboratories has occurred (75, 140).

Plugging of systems for the secondary recovery of oil.—Here their activities are not in dispute, though they are by no means the only organisms involved; the question has been the subject of a recent symposium (134).

Spoilage of oily materials.—Spoilage of stored petroleum fuels in tropical areas, leading to an increase in the sulphur content of the nonaqueous phase has been attributed primarily to the growth of sulphate-reducing bacteria in water at the bottom of petroleum storage tanks (30, 31). This phenomenon has led to serious strategic and economic difficulties owing to the grounding of aircraft. Sulphate-reducing bacteria have also been held as contributing to the spoilage of cutting emulsions when the material is stagnant (17).

Spoilage of canned food.—Clostridium nigrificans, the thermophilic sulphate-reducing organism, is the causative agent of "sulphur-stinker" spoilage of canned foods (143).

Pollution of waters.—Butlin (20) reviewed water pollution by these bacteria and methods for its control. Some success in field experiments has been achieved by adding chromate to inhibit the growth of these bacteria specifically (38); Bunker's (19) acidification procedure has shown promise in field experiments with oil well waters (4). In nature, large-scale growth of

these organisms in the sea can lead to ecological disasters such as the mass mortality of fish (34) or damage to rice fields [see (58)].

Blackening of paper pulp.—Reduction of sulphates and sulphites in paper pulp can lead to blackening caused by precipitation of FeS (15).

Contamination of town gas—Growth of the bacteria in gas holder water can lead to contamination of town gas with H₂S (118).

Recycling bound phosphate.—The productivity of inland waters is dependent on the circulation of phosphate. Bacterial sulphate-reduction is believed to cause the release of soluble phosphate from basic ferric phosphates by promoting their conversion to FeS (48, 125).

Formation of sulphate-deficient waters.—Beauchamp (14) attributed sulphate-deficient waters and soils in Africa to the activities of sulphate-reducing bacteria, but the work of his colleague, Hesse (49), indicates that this is not so.

Nutrition of ruminants.—Sulphate-reducing bacteria are found in the rumens of cattle (47) and sheep (76); in the latter they are believed to be of nutritional importance in providing reduced sulphur for biosyntheses of protein by the sheep (3).

Formation of "medicinal" muds.—The hot muds of Piestany, Czechoslovakia, to which medicinal properties are attributed, are "ripened" by the action of the thermophilic sulphate-reducing bacteria (127).

Separation of sulphur isotopes.—The optimum conditions for this process, first demonstrated by Thode and his colleagues (80, 136), have been established by Jones & Starkey (65).

Progenesis of living material.—The evidence that sulphate-reduction has been detectable up to 8×10^8 years ago (137), together with the demonstration that they possess cytochrome systems analogous to those of aerobes, underlines the possibility suggested by van Niel (90) that they share with the photosynthetic anaerobes the evolutionary ancestry of air-breathing organisms.

These variegated economic activities have generated proposals for the exploitation of sulphate-reducing bacteria. Suggestions for the "farming" of sulphur (133), for the deliberate infection of sulphur springs (25), and for the production of sulphur from sewage sludge (27) have arisen from their role in the formation of elemental sulphur and the latter process is now at a pilot-plant stage (32). Sulphate is one of several possible electron acceptors for the biological treatment of fluid wastes (83); a process for the treatment of yeast wastes with these bacteria has been worked in Czechoslovakia (43). Their use for the removal of sulphite from waste liquor in paper manufacture has been proposed (12); bacterial fermentation of sulphite waste liquor for the recovery of sulphur would appear to be uneconomic (81).

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